In science we resemble childeren collecting a few pebbles at the beach of knowledge, while the wide ocean of the unknown unfolds itself in front of us

In de wetenschap gelijken wij op kinderen, die aan de oever der kennis hier en daar een steentje oprapen, terwijl de wijde oceaan van het onbekende zich voor onze ogen uitstrekt

Sir Isaac Newton

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Chlamydia psittaci: epidemiology, zoonosis and examination of the innate immunity

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Table of contents

Abbreviations

ACC	Atypical chicken *Chlamydiaceae*
ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine di-phosphate
ANOVA	Analysis of variance
AP-1	Activator protein 1
ARDS	Adult respiratory distress syndrome
ATP	Adenosine tri-phosphate
BGM	Buffalo green monkey
BSA	Bovine serum albumin
BSL3	Biosafety level 3
C.	*Chlamydia*
CD	Cluster of differentiation
CFT	Complement fixation test
COMC	*Chlamydia* outer membrane complex
Cop	*Chlamydia* outer protein
CPAF	Chlamydial protease-like activity factor
CRP	Cysteine rich protein
Ct	Cycle treshold
CTL	Cytotoxic T cell
DAMP	Damage-associated molecular pattern
DAPI	4',6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's minimal essential medium
dpi	Days post infection
EB	Elementary body
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activating-cell sorting
FAM	5-carboxyfluorescein
FCS	Fetal calf serum
GAG	Glycosaminoglycan
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPIC	Guinea pig inclusion conjunctivitis
HSP	Heat shock protein

IB	Intermediate body
ICSP	International committee on systematic prokaryotes
iE-DAP	D-γ-glutamyl-meso-DAP dipeptide
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFU	Inclusion forming unit
IL	Interleukin
IM	Inner membrane
Inc	Inclusion membrane protein
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
ISCOM	Immunostimulating complexes
LITAF	Lipopolysaccharide-induced tumor necrosis alpha factor
LPB	LPS binding protein
LPS	Lipopolysaccharide
LGV	Lymphogranuloma venereum
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
MFA	Mean fluorescence area
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIF	Micro Immuno Fluorescence
MIP	Macrophage infectivity potentiator
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MoPn	Mouse Pneumonitis
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MyD88	Myeloid differentiation 88
N_0	Initial template quantities
NF-κB	Nuclear factor kappa beta
NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain containing 3

NOD	Nucleotide-binding oligomerization domain
OEA	Ovine enzootic abortion
OmcA	Outer membrane complex A
OmcB	Outer membrane complex B
OmpA	Outer membrane protein A
OvoTF	Ovotransferrin
PAI	Pathogenicity island
PAMP	Pattern associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
pi	Post infection
PMA	Phorbol 12-myristate 13-acetate
POMP	Polymorphic outer membrane protein
PorB	PorinB
PPD	p-Phenylenediamine
PRR	Pathogen recognition receptor
RB	Reticulate body
RIG	Retinoic acid-inducible gene
RIP2	Receptor-interacting protein 2
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
ROS	Reactive oxygen species
Sct	Secretion and cellular translocation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error mean
siRNA	Small interfering ribonucleic acid
SPF	Specific pathogen free
STD	Sexually transmitted disease
T3SS	Type III secretion system
T3S	Type III secretion
TAMRA	N,N,N,N’-tetramethyl-6-carboxylrhodamine
Tarp	Translocated actin-recruiting phosphoprotein

TCA	Tricarboxylic acid
TCID	Tissue culture infective dose
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
THP1	Monocytic leukemia cell line
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Treg	T regulatory cell
TRIF	Tir-domain-containing adaptor inducing interferon β
TRIR	Total RNA Isolation Reagent
tRNA	Transfer ribonucleic acid
TWAR	Taiwan acute respiratory agent
US	United States
Vero	Africa Green Monkey
WT	Wild-type

Study objectives

The present study focuses on *Chlamydia psittaci* (*C. psittaci*), which is an obligate intracellular Gram-negative bacterium causing respiratory disease and mortality in many bird species (Kaleta & Taday, 2003). *C. psittaci* can be transmitted through inhalation of aerosols of nasal secretions and feces from infected birds (Burkhart and Page, 1971; Cole, 1990). Initially, the respiratory tract is colonized, from where the disease further spreads to other organs, leading to a systemic infection. *C. psittaci* is responsible for huge economical losses in the poultry industry resulting from lowered egg-production and the need of antibiotic treatment (Grimes & Wyrick, 1991). Moreover, *C. psittaci* can be transmitted to humans causing psittacosis, the clinical outcome ranging from inapparent to severe flu-like symptoms or pneumonia (Andersen and Vanrompay, 2000). Especially, people in close contact with birds such as poultry workers, pet bird owners and veterinarians are at risk for this zoonotic agent.

The **first objective** of this thesis was: a) to determine how widespread *C. psittaci* and atypical *Chlamydiaceae* (ACC) were among chickens in Flanders and b) to investigate their zoonotic transmission.

After infection, the primary replication of *C. psittaci* takes place in the epithelial cells and macrophages of the respiratory tract. Subsequently, the bacteria can be found in macrophages of the vascular system. It is believed that *C. psittaci* uses these macrophages as a vehicle to spread throughout the body to start replication in epithelial cells and macrophages of various tissues and establish a systemic infection. Because *C. psittaci* can survive and even replicate within a cell which is part of the innate immune system, the macrophage, and the fact that this bacteria is widespread among chickens, the **second objective** of this thesis was to examine the innate immune response elicited by *C. psittaci* in chicken macrophages.

As *C. psittaci* possesses a huge zoonotic potential, the **third objective** aims to determine the relationship between *C. psittaci* and the human macrophage, with the emphasis on internalization, survival and replication and on the innate immune system.

Treatment with antibiotics can easily resolve a *C. psittaci* infection. However, tetracycline resistance has already been reported for *C. suis* through horizontal gene transfer (Dugan et al., 2004; 2007). Antibiotics, especially tetracyclines are widespread in the poultry industry, creating the risk for tetracycline resistance among *C. psittaci* strains. This calls for research on creating an effective vaccine against *C. psittaci*. Therefore, a good understanding is needed of the mediators, the PRRS and the cells involved in the host immune response during a *C. psittaci* infection and how these factors interact with

each other. The **fourth objective** of this thesis was to investigate the *in vivo* innate immune response evoked by *C. psittaci* in its avian host, the chicken.

Chapter I

Chlamydial Infection Biology

1. Introduction

Chlamydia psittaci (*C. psittaci*) is an obligate intracellular bacteria causing respiratory disease in a wide variety of domestic and wild birds (Grimes, 1994). It has been shown , that *C. psittaci* is endemic in many species of wild birds, but also in poultry like chickens, turkeys, geese and ducks (Newman et al., 1992; Vanrompay et al., 1997b; Verminnen et al., 2008; Lagae et al., 2014). Furthermore, this bacterium is a zoonosis that causes psittacosis in humans. As transmission occurs through inhalation of pathogen-containing aerosols from faeces and nasal tract exudates, people in close contact with birds are at risk like farmers, pet keepers, employees of abattoirs and taxidermists.

2. History

In 1879, Jacob Ritter associated for the first time a strange form of pneumonia in Switzerland with the importation of infected psittacine birds (Ritter, 1880). Three years later, after an outbreak in Paris, the disease was called 'psittacosis' after the Greek word for parrot 'psittakos' as parrots showed to be the source of the infection (Morange, 1895).

Halberstaedter and van Prowazek described for the first time in 1907 intracytoplasmatic inclusions containing a large number of micro-organisms derived from conjunctival scraping of patients suffering from trachoma. Because a classical Giesma staining showed a blue matrix or mantle surrounding the particles, these organisms were wrongly assumed to be protozoa, named 'Chlamydozoa' after the Greek word 'Chlamys' for mantle (Halberstaedter and von Prowazek, 1907a; Halberstaedter and von Prowazek, 1907b). However, later studies revealed that these micro-organisms were bacteria instead of protozoa. This was the beginning of a long and confusing process of chlamydial classification.

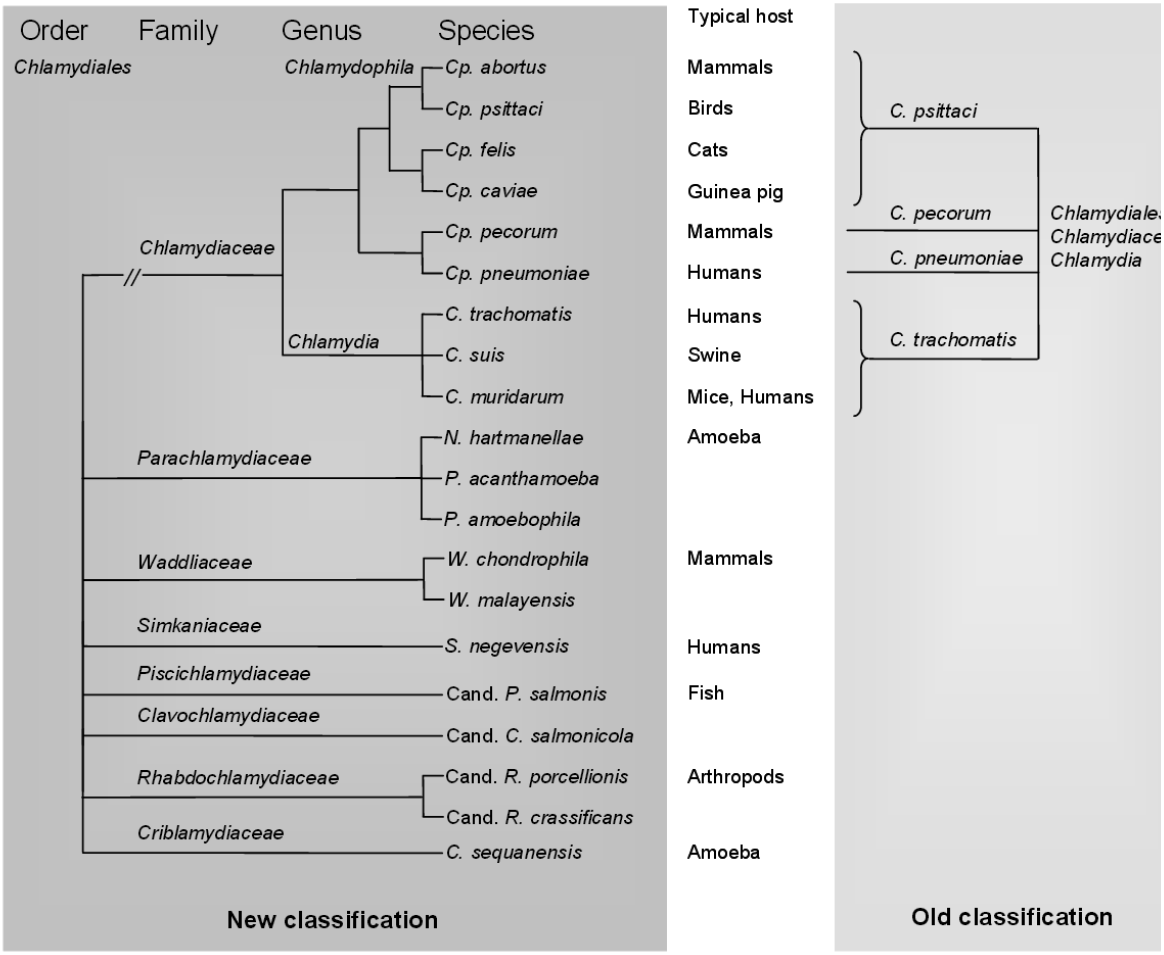
During the winter of 1929-1930, a pandemic outbreak of psittacosis was reported due to the shipment of green Amazon parrots from South America to the United States and Europe (Coles, 1930). Bedson and Western managed successfully to isolate the causative agent in 1930 (Bedson and Western, 1930). Until 1930, it was commonly believed that only psittacine birds were the source of avian chlamydiosis infections. Although, later on, other animals like chickens, pigeons and ducks were shown to transmit psittacosis to humans (Meyer and Eddie, 1932; Pinkerton and Swank, 1940; Wolins, 1948). In the same period, researchers were able to isolate the causative agent of lymphogranuloma venereum (LGV) from human tissue, which is a serious genital infection (Hellerström & Wassén, 1930). Both particles were classified as viruses belonging to the psittacasosis-LGV group. Two years later, the developmental cycle, like it is known today, was for the first time described. Beside the previously identified elementary body, a novel form of the organism was discovered: the reticulate body (Bedson and Bland, 1932).

Finally, 20 years after the first classification, in 1966, *Chlamydiae* were recognized as Gram-negative bacteria. Because of new techniques like electron microscopy and cell culture, research revealed that these organisms have a Gram-negative-like envelope, a distinct developmental cycle with EBs and RBs containing both DNA and RNA and prokaryotic ribosomes susceptible to antibiotics (Moulder, 1966). The organisms causing psittacosis, LGV and trachoma were combined into one single genus: *Chlamydia* (Page, 1966).

3 Taxonomy

Originally, the order *Chlamydiales* has one family, the *Chlamydiaceae*, containing one genus, *Chlamydia* and just two species, *Chlamydia trachomatis* and *Chlamydia psittaci* (Page, 1968). Due to the development of DNA-based classification methods during the 1980s, two additional species, *Chlamydia pneumonia* (Grayston et al., 1989) and *Chlamydia pecorum* (Fukushi and Hirai, 1992) were introduced. In 1999, Everett *et al.* (1999) proposed a new classification based on phylogenetic analyses of 16S and 23S rRNA genes, and on phenotypic, morphologic and genetic information. This information showed that the order *Chlamydiales* contains at least four distinct groups at the family level: the *Chlamydiaceae*, the *Parachlamydiaceae*, the *Simkaniaceae* and the *Waddliaceae*. The *Chlamydiaceae* were divided into two genera, *Chlamydia* and *Chlamydophila*, comprising nine species (Everett et al., 1999). The order of *Chlamydiales* was further extended with four new families of *chlamydia*-like bacteria, *Piscichlamydiaceae*, *Clavochlamydiaceae*, *Rabdochlamydiaceae* and *Criblamydiaceae* (Horn, 2008) (Fig. I-1).

Fig. I-1: Overview of the new classification as proposed by Everett et al. (1999) versus the old classification (adapted from Bush and Everett, 2001).



However, regarding the taxonomy proposed by Everett *et al.*, there are still a lot of issues that need to be addressed. One of them is the proposal to split the genus *Chlamydia* in two genera, *Chlamydia* and *Chlamydophila*, which has led to significant controversy (Schachter et al., 2001; Stephens et al., 2009). Due to these issues, a subcommittee of the International committee on systematic prokaryotes (ICSP) on the taxonomy of *Chlamydiae* was formed in 2009. This subcommittee decided that a single genus, *Chlamydia*, will be used. To support this proposal, a working group will analyze the data derived from all recently available genome sequences of the members of the family *Chlamydiaceae*. The new classification will be applied in this thesis.

Recently, a new member of *Chlamydiaceae*, named atypical *Chlamydiaceae* (ACC), was isolated from a group of poultry slaughterhouse workers in France who suffered from atypical pneumonia. Phylogenetic analysis based on 16S rRNA showed a seperate position of ACC among other *Chlamydia* species within the family of *Chlamydiaceae* (Zocevic et al., 2012).

3.1. Chlamydiaceae

Today, the *Chlamydiaceae* have one genus, *Chlamydia* with nine species (Table I-1). The nine species differ in inclusion morphology, pathogenicity and host specificity (Everett *et al.,* 1999).

Table I-1: The family *Chlamydiaceae* (Kerr *et al.,* 2005)

Species	Host	Clinical signs
Chlamydia trachomatis	Humans	Chronic conjunctivitis and blindness (trachoma) Sexually transmitted disease (STD) Infection of the urogenital tract, infertility
Chlamydia muridarum[c]	Mice Hamsters	Respiratory tract infection Genital tract infection
Chlamydia suis[c]	Pigs	Diarrhea, pneumonia, conjunctivitis, reproductive disorders
Chlamydia pneumoniae	Humans Koala	Pneumonia, bronchitis, encephalomyelitis, laryngitis, atherosclerosis, reactive arthritis
Chlamydia psittaci[a]	Humans Birds	Respiratory tract infection
Chlamydia abortus[a]	Ruminants Pigs	Reproductive disorders, abortion and bad semen quality
Chlamydia pecorum[c]	Ruminants Pigs Koala	Reproductive disorders, infertility, infection of the urine tract (koala) and abortion, enteritis, polyarthritis, encephomyelitis, metritis, conjunctivitis and pneumonia (other animals)
Chlamydia felis[b]	Cats	Conjunctivitis and respiratory tract infections
Chlamydia caviae[b]	Guinea pigs	Ocular and urogenital tract infection

[a]Zoonotic pathogen (multiple case reports)
[b]Zoonotic pathogen (a few case reports)
[c]Zoonotic potential cannot be excluded (not yet investigated)

3.1.1. Chlamydia trachomatis

C. trachomatis infections are the leading cause of bacterial sexually transmitted disease (STD) in industrialised countries and of infectious blindness (trachoma) in Africa, the Middle East, Asia and South-America. STDs caused by *C. trachomatis* are strongly associated with urethritis, cervicitis, pelvic inflammatory disease and, at later stage, chronic pelvic pain, ectopic pregnancy and tubal factor infertility in women. In men, symptoms include urethritis, epididymitis, prostatitis and infertility. In both sexes, it can cause mucosal inflammation of the throat and rectum (Spiliopoulou *et al.,* 2005). *C. trachomatis* is divided in two biovars: trachoma and lymphogranuloma venereum (LGV) (Longbottom and Coulter, 2003). The trachoma biovar is comprised of 14 serovars (A to K, Ba, Da and Ia) which

primarily infects columnar epithelial cells of mucous membranes: serovars A to C were specially associated with trachoma, while D to K were associated with sexually transmitted infection (Wang and Grayston, 1970). The LGV biovar consisted of four serovars (L1, L2, L2a and L3) which proliferated in lymphatic and subepithelial tissues and caused systemic infections (Schachter, 1999). The reference strains for the trachoma and LGV biovar are C/PK-2 and L2/434/BU (ATCC VR 902B), respectively.

3.1.2. *Chlamydia muridarum*

Two strains of *C. muridarum*, MoPn and SPFD have been respectively isolated from mice and hamsters (Fox et al., 1993; Stills, 1991). MoPn (mouse pneumonitis) was isolated in 1942 from the lungs of asymptomatic albino Swiss mice and was subsequently shown to be capable of producing pneumonia in mice (Nigg et al., 1942). MoPN have been shown to be sensitive to sulfadiazine and harbors and extrachromosomal plasmid (Moulder et al., 1965). It is also the type strain for *C. muridarum* (ATCC VR 123^T). SPFD obtained from a hamster, is known to cause asymptomatic infection (Fox et al., 1993).

3.1.3. *Chlamydia suis*

C. suis has only been isolated from swine, in which it may be endemic (Zahn et al., 1995; Szeredi et al., 1996; Schiller et al., 1997) causing conjunctivitis, enteritis, pneumonia and a high incidence of apparently asymptomatic infections (Rogers et al., 1996; Rogers and Andersen, 1996; Rogers and Andersen, 1999). *C. suis* often appears as a mixed infection with *C. abortus* (Hoelzle et al., 2000). Several strains of *C. suis* are known to have an extrachromosal plasmid, pCS. Some strains have enhanced resistance to sulfadiazine and/or tetracycline, which is alarming for human health as *C. suis* is closely related to *C. trachomatis* (Everett, 2000; Lenart et al., 2001). Isolate S45^T (ATCC VR 1474^T) is the reference strain.

3.1.4. *Chlamydia pneumoniae*

C. pneumoniae is comprised of three biovars, one human Taiwan acute respiratory agent (TWAR) and two animal biovars, Koala and Equine infecting horses and frogs respectively (Berger et al., 1999). TWAR causes about 10% of all community-acquired pneumonia in adults and 5% of bronchitis and sinusitis (Grayston et al., 1993). It is the third most common cause of pneumoniae and most infections are asymptomatic, especially in children. Transmission is person to person by respiratory particles. Furthermore, it has been associated with atherosclerosis (Grayston, 1999; Sessa et al., 2009), Alzheimer's disease (Balin et al., 1998; Honjo et al., 2009), asthma (Clementsen et al., 2002; Newcomb and Peebles, Jr., 2009) and multiple sclerosis (Stratton and Wheldon, 2006). The Koala biovars isolates are commonly obtained from ocular and urogenital sites. All five Australian free-ranging koala populations are infected with *C. pneumonia* inducing respiratory illness (Everett, 2000). The Equine

biovar includes one respiratory isolate, N16, isolated from a nasal swab of a horse and contains an extrachromosomal plasmid (Wills et al., 1990). The type strain for *C. pneumoniae* is TW-183^T (ATCC VR 2282^T).

3.1.5. *Chlamydia pecorum*

C. pecorum has been isolated from mammals like ruminants (cattle, sheep, goats) (Fukushi and Hirai, 1992), koalas (Girjes et al., 1993) and swine (Kaltenbroeck and Storz, 1992; Anderson et al., 1996). In koalas, clinical manifestations of *C. pecorum* include ocular infection leading to conjunctival scarring and blindness, respiratory tract infections, urinary tract infections causing incontinence, and genital tract infections potentially leading to infertility. In other animals, *C. pecorum* causes abortion, encephalomyelitis, conjunctivitis, pneumonia, enteritis and polyarthritis. The reference strain is E58^T (ATCC VR 628^T).

3.1.6. *Chlamydia abortus*

C. abortus is endemic among sheep, goats and ruminants and has been associated with abortion, premature or full-term delivery of stillborn, moribund or weakly animals failing to survive beyond 48h. The disease among sheep is also known as ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE). OEA is recognized as a major cause of lamb loss in sheep throughout Europe and is encountered in most sheep-rearing areas of the world, including Africa and North-America. Cattle, pigs and horses are also infected, but it is thought to occur to a much lesser extent (Longbottom and coulter, 2003). *C. abortus* is well known as a zoonotic pathogen with the biggest threat to pregnant women, because of the ability of *C. abortus* to colonize the placenta. The outcome of human infection in the first trimester of pregnancy is likely to be spontaneous abortion, whereas later infection causes stillbirths or preterm labour (Hyde and Benirschke, 1997). The type strain for *C. abortus* is B577^T (ATCC VR 656^T).

3.1.7. *Chlamydia felis*

C. felis is endemic among domestic cats worldwide, primarily causing inflammation of feline conjunctivae, rhinitis and respiratory problems (Gaillard et al., 1984). Transmission requires close contact between cats and ocular secretions are the most important body fluid for infection (Gruffyd-Jones et al., 2005). There is evidence that *C. felis* may occasionally cause keratoconjunctivitis in humans and sporadically pneumonia, due to close contact between cats and their owners (Hartley et al., 2001; Browning, 2004). The type strain for *C. felis* is FP Baker^T (ATCC VR 120^T).

3.1.8. *Chlamydia caviae*

C. caviae primarily infects the mucosal epithelium of the conjunctivae of guinea pigs, resulting in ocular inflammation and eye discharge. It can also infect the genital tract of guinea pigs where the disease has many similarities with a human genital infection. There are six strains of *C. caviae* or guinea pig inclusion conjunctivitis (GPIC) isolated from guinea pigs, which share an identical *ompA* sequence (Zhao et al., 1993). The reference strain for *C. caviae* is GIPC^T (ATCC VR 813^T), which harbors an extrachromosomal plasmid pCpGP1 (Lusher et al., 1989).

3.1.9. *Chlamydia psittaci*

C. psittaci causes worldwide respiratory diseases in pet birds and poultry. The infection is often systemic and can be unapparent, severe, acute or chronic with intermittent shedding. The symptoms include conjunctivitis, anorexia, nasal discharge, rhinitis, diarrhea, polyuria, dyspnea and dullness (Vanrompay et al., 1995a). Transmission of *C. psittaci* occurs by inhalation of aerosols created from respiratory tract exudates and faecal material of birds. The transmission can also be vertical through the egg (Wittenbrink et al., 1993). *C. psittaci* compromises nine known genotypes (A-F, E/B, M56 and WC) and is a well-known zoonotic pathogen. In humans, the disease can vary from mild flu-like symptoms to severe pneumonia (Beeckman and Vanrompay, 2009). Several strains harbor an extrachromosomal plasmid. The reference strain for *C. psittaci* is 6BC^T (ATCC VR 125^T). A more detailed discussion can be found in paragraph 6 and 7 of this chapter.

3.1.10. *Atypical Chlamydiaceae*

In 2008, atypical *Chlamydiaceae* were detected for the first time in chickens during a psittacosis outbreak in Germany (Gaede et al., 2008). Subsequently, they were isolated from chickens in France, while atypical pneumonia were simultaneously reported among slaughterhouse workers in France (Larouceau et al., 2009). In 2012, ACC have also been detected in Australian, German, Greek, Croatian, Slovenian and Chinese chicken flocks (Robertson et al., 2010; Zocevic et al., 2012). These data suggest that ACC could be distributed worldwide among chickens. Interestingly, atypical strains of *Chlamydiaceae* were also indentified in turkeys and pigeons (Gasparini et al., 2011).

3.2. *Parachlamydiaceae*

The *Parachlamydiaceae* are *Chlamydia*-like endosymbionts that naturally infect free-living amoebae, specifically *Acanthamoeba* and *Hartmanella* (Amann et al., 1997; Horn et al., 2000). They have a *Chlamydia*-like cycle of replication and 80% to 90% homology of ribosomal genes. The *Parachlamydiaceae* family compromises two genera, of which the type strains are the *Parachlamydia*

acanthamoebae and the *Neochlamydia hartmanellae* (Greub and Raoult, 2002). It has been shown that *P. acanthamoebae* can induce respiratory disease in humans (Marrie et al., 2001). Moreover, experimental models have demonstrated the ability of *P. acanthamoebae* to enter and replicate within human macrophages (Greub et al., 2005) and pneumocytes (Casson et al., 2006) and to cause pneumonia in mice (Casson et al., 2008). In 2008, *Parachlamydiaceae* was linked to bovine abortion cases in Switzerland as the organism was detected by 16S rRNA PCR and within placental lesions by immunohistochemical analyses. Thus caution should be taken when handling bovine abortion material because of the potential zoonotic risk (Ruhl et al., 2008). Genome analysis showed that strain UWE 25 contains no homologues of the major outer membrane protein (MOMP) or of the family of polymorphic outer membrane proteins (POMPs), but it does encode a complete Type III secretion system (T3SS) and some effector proteins (Horn et al., 2004).

3. 3. *Waddliaceae*

The family of the *Waddliaceae* comprises a single genus, the type genus *Waddlia* and two species. The first species, *Waddlia chondrophila* currently includes only the type strain, WSU 86-1044^T (ATCC VR 1470^T), which was isolated from the tissues of a first-trimester aborted bovine fetus (Dilbeck et al., 1990). *Waddlia* was also demonstrated by PCR and serology in women with sporadic and recurrent miscarriages. *Waddlia* seropositivity was associated with animal contact suggesting that *Waddlia* may be zoonotically transmitted, but infection through contaminated water, uncooked meat, milk or sexual contact might also be the mode of transmission to humans (Corsaro et al., 2002; 2003; Baud et al., 2007; 2008). In 2005, the second species, *Waddlia malayensis* was isolated from urine samples from fruit bats in Northern Malaysia. They are resistant to penicillin and streptomycin, but sensitive to tetracyclin (Chua et al., 2005).

3. 4. *Simkaniaceae*

The family *Simkaniaceae* currently includes two genera: *Simkania* and *Fritschea*. The type species is *Simkania negevensis*, originally discovered as contaminant in a human cell culture (Corsaro et al., 2003; Kahane et al., 1995). Its original host is unknown, but it grows well in *Acanthamoeba* spp. and other amoeba (Kahane et al., 2001). On the basis of molecular and serological data, *Simkania negevensis* has been associated with bronchiolitis and community-acquired pneumonia in children and with exacerbation of chronic obstructive pulmonary disease in adults (Kahane et al., 1998; Lieberman et al., 1997; 2002). *Simkania negevensis* has a longer developmental cycle when compared to other chlamydiales (7-12 versus 2-3 days) (Kahane et al., 1999; 2002). The type strain Z^T does not possess an extrachromosomal plasmid (Everett et al., 2000). *Fritschea* are endosymbionts of the plant-feeding whitefly *Bemisia tabaci* and scale insect *Eriococcus spurius*. In the gut of *Bemisia tabaci*, these

bacteria live within bacteriocytes transmitted directly from the parent to the oocytes (Everett et al., 2005).

4. Morphology

4. 1. Developmental forms

Chlamydiaceae are obligate intracellular pathogens with a unique biphasic developmental cycle, in which it alternates between two distinct morphological forms, the small extracellular infectious elementary body (EB) and the intracellular, non-infectious, metabolically active reticulate body (RB), as well as by intermediate forms (IBs) (Vanrompay et al., 1996).

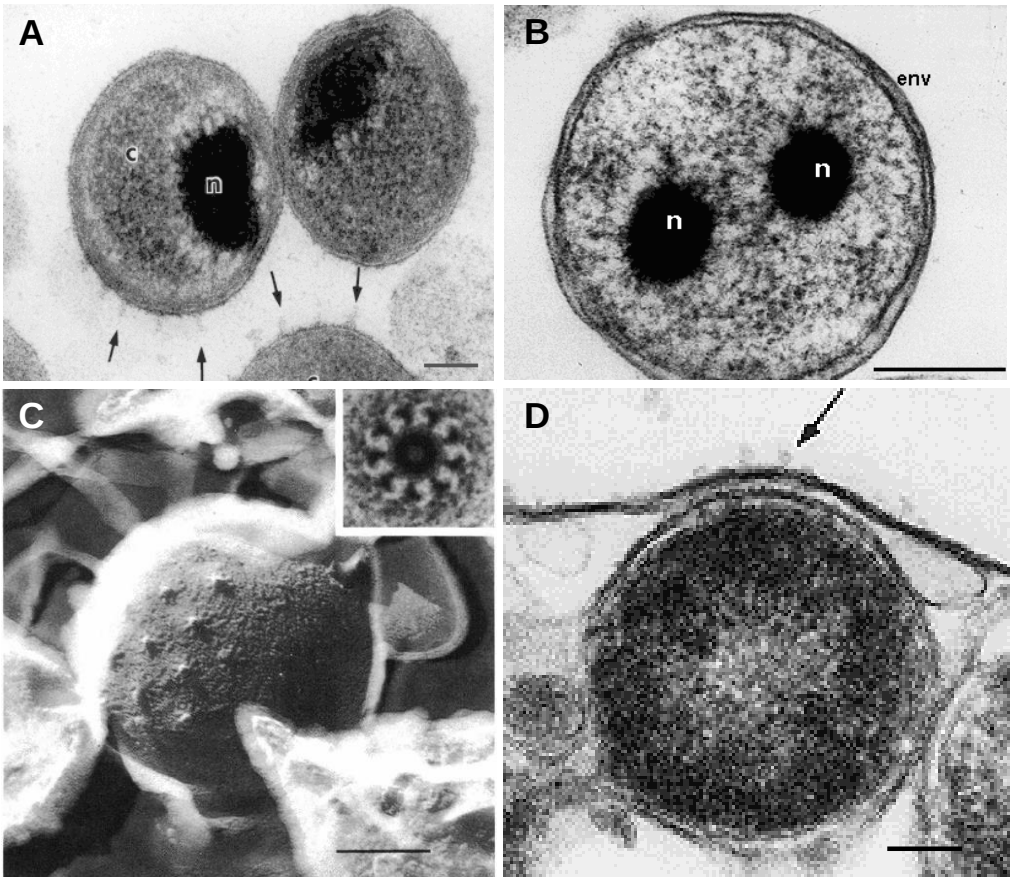
Elementary bodies are small, spherical or occasionally pear shaped electron-dense structures with a diameter of 0.2-0.3 µm. It is characterized by a dense, eccentric core of condensed DNA and chromatin and it has a granular appereance due to the presence of 70S ribosomes (Fig. I-2A/C). The EB is the only infectious stage of the chlamydial developmental cycle. It functions as a tough 'spore-like' body whose purpose is to permit chlamydia survival in the non-supportive environment outside the host cell. This resistance to both physical and chemical factors in the extracellular environment is a consequence of the rigidity of the cell envelope, which is both osmotically stable and poorly permeable, and also of the greatly reduced surface area of the EB (Longbottom and Coulter, 2003). The EB is thought to be metabolically inert until it attaches to, and is endocytosed by, a susceptible host cell. It contains only small amounts of the bacterial cell wall strengthening substance, peptidoglycan (Everett et al, 1994). Instead it derives its strength from cross links (S-S bridges) formed between the sulphur atoms of its sulphur amino acids (cysteine and methionine) rich proteins in the outer envelope, the cysteine rich proteins (Newhall and Jones, 1983).

The non-infectious **reticulate bodies** have a diameter of 0.5-1.6 µm and are responsible for intracellular replication. They are metabolically active and as a consequence their cytoplasm is rich in ribosomes and RNA, which are required for protein synthesis (Longbottom and Coulter, 2003). They possess a fragile membrane lacking the extensive disulfide bonds characteristic of the EB, resulting in a more permeable membrane to facilitate nutrient uptake (Newhall and Jones, 1983). Their nucleoid acid appears diffuse and fibrillar. The differentiation of EB to RB involves an expansion to a diameter ranging from 0.5 to 1.6 µm, resulting in a less electron dense cytoplasm in which the nucleus can no longer be distinguished (Fig. I-2D).

During the developmental cycle, RBs begin to differentiate again into EBs inside the chlamydial inclusion. The initial sign of this differentiation is the re-condensation of chlamydial nucleic acid in to histone protein. This stage is called the **intermediated body** with a diameter of 0.3 to 1.0 µm and a

central electron dense core (Fig. I-2B) (Litwin et al., 1961; Costerton et al., 1976; Vanrompay et al., 1996).

Fig. I-2: Overview of *C. psittaci* and *C. trachomatis* developmental forms.



A. Transmission electron microscopic picture of a section throough an EB of *C. psittaci*. Note the projections extending from the outer membrane. The bar represents 0.15 µm. Adapted from Matsumoto (1988).
B. An intermediate body of *C. trachomatis* undergoing differentiation. Two points of condensation of DNA into electron dense nucleoids (n) can be observed as well as the inner cytoplasmic membrane and the outer envelope (env). The bar represents 0.25 µm. Adapted from Ward (1988).
C. Stereographic viewing of a purified EB surface. The bar represents 0.1 µm. Adapted from Matsumoto (1982)
D. Transmission electron microscopic picture of a section through an RB of *C. psittaci*. Surface projections penetrating the inclusion membrane can be observed. The bar represents 0.1 µm. Adapted from Matsumoto (1988).

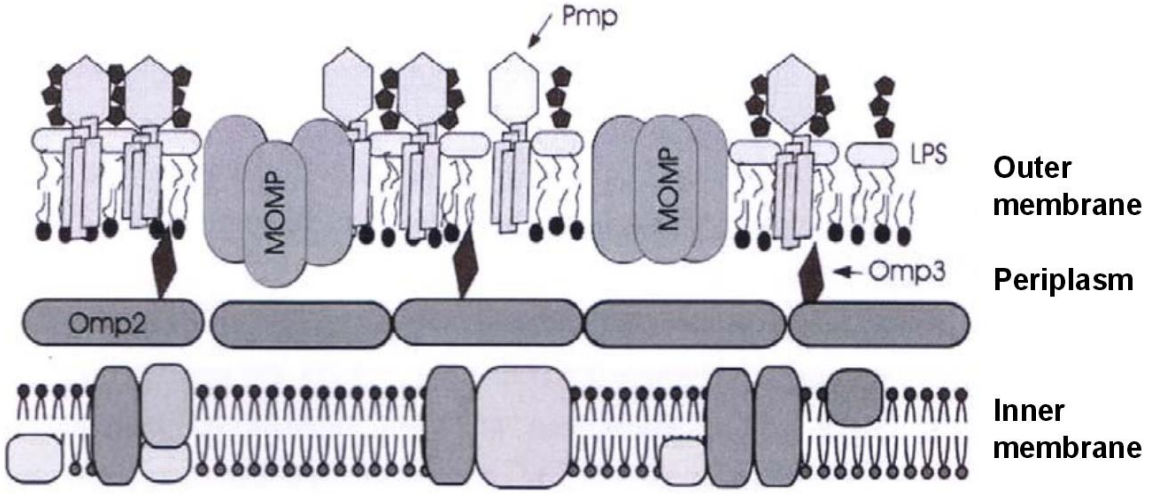
4. 2. Outer membrane composition

The cell envelope of *Chlamydiaceae* consists of a cytoplasmatic inner membrane and an outer membrane separated by a periplasmatic space. The outer membrane contains proteins, phospholipids, lipopolysaccharides (LPS) and lipids. The most striking feature of the chlamydial envelope is the presence of negligible amounts of peptidoglycan (Hatch, 1996; Moulder, 1993). This is in contrast to other Gram-negative bacteria where a part of the cell wall contains peptidoglycan, covalently bound

to lipoproteins, which is insoluble in ionic detergents like sacrosyl. However, a part of the cell wall of *Chlamydiaceae* is still insoluble in ionic detergents, called the Chlamydia Outer Membrane Complex' or COMC. This complex consist of the 'Major Outer Membrane Protein' (MOMP), Polymorphic membrane proteins (Pmps) and two cysteine rich proteins (CRP): Outer membrane complex A protein (OmcA or Omp3) and Outer membrane complex B protein (OmcB or Omp2) (Hatch et al., 1984; Sardinia et al., 1988; Stephens et al., 2001). The outer membrane is further composed of Omp85, PorB, LPS and the heat shock proteins hsp60 and hsp70. A schematic overview of the chlamydial envelope is given in Figure I-3.

A combination of the double-layer membrane and efficient efflux pumps present in *Chlamydiaceae* prevent effective translocation across the membrane of these bacteria. Multidrug efflux mechanisms are broadly conserved in bacteria. In *Chlamydia spp.*, efflux is the predominant mechanism of tetracycline resistance (Dugan et al., 2004; Pool, 2005).

Fig. I-3: Schematic overview of the envelope of chlamydial EBs. In the outer membrane, MOMP, both CRPs and the pmps are shown. Specific proteins of the inner membrane are not yet identified (Adapted from Hatch, 1996).



4.2.1. Major outer membrane protein

The chlamydial major outer membrane protein (MOMP) was discovered in 1981 by three independent laboratories, due to relatively new techniques of SDS-PAGE electrophoresis (Hatch et al., 1981; Caldwell et al., 1981; Salari and Ward, 1981). The 40 kDa MOMP is one of the predominant proteins at the surface of EBs and RBs, where it covers 60% of the total outer membrane protein (Caldwell et al., 1981). MOMP is critical for its chlamydial function because it functions as a structural protein (Hatch, 1996), a general porin (Bavoil et al., 1984; Wyllie et al., 1998; Jones et al., 2000) and a potential chlamydial cytoadhesin (Su et al., 1990; Swanson and Kuo, 1994). The most widely studied and

accepted function of MOMP is that of a porin. Exposure to thiol reducing agents lead to reduction in disulfide bridges, opening up its pore structure for the uptake of nutrients and partly triggering the early differentiation of EBs after entry in the host cell. These porin characteristics were also confirmed by electrical studies of native and recombinant MOMP in planar lipid bilayers (Wyllie et al., 1998; 1999). Like other bacterial porins, MOMP has predominantly β-sheet content (62%) (Wyllie et al., 1998). Four variable domains (VD1 to 4), located on the outer loops (i.e. exposed on the surface) are flanked by highly conserved regions (Baehr et al., 1988; Yuan et al., 1989). These variable domains contain genus-, species-, subspecies- and serovar-specific antigenic determinants (Caldwell et al., 1981; Everett, 2000; Kim and DeMars, 2001). The serovar- and subspecies-specific epitopes are surface-exposed and act as targets for neutralizing antibodies, while the species- and genus-specific epitopes are inaccessible to antibodies and therefore non-neutralizing (Batteiger et al., 1986; Kuo and Chi, 1987; Zhang et al., 1987). Since MOMP is immunodominant and generates host antibodies which are protective, there was much interest in MOMP as a candidate for vaccine development.

4. 2. 2. Cystein-rich proteins: OmcA and OmcB

Apart from MOMP, the major proteins in the COMC are the two cysteine-rich proteins (CRPs) OmcA and OmcB. OmcB or Omp2 is the largest of the two CRPs with a molecular weight of 60 kDa. The amino acid sequence reveals a protein containing 24 cysteine residues, where the position of these residues is highly conserved (Allen and Stephens, 1989). OmcB is highly conserved among chlamydia species. It is a structural protein involved in the conversion of the RB to the EB (Mygind et al., 1998) and believed to contribute to the cell-wall rigidity and osmotic stability of the EB (Newhall, 1987). Furthermore, it is highly immunogenic (Sanchez-Campillo et al., 1999), carries genus-specific epitopes (Watson et al., 1994) but it is thought not to be surface accessible to antibody binding (Watson et al., 1994; Everett and Hatch, 1995). OmcB seems to be located in the periplasm (Everett and Hatch, 1995). However, a few reports have suggested that it plays a role in attachment and invasion of the host cell (Ting et al., 1995; Stephens et al., 2001). Later studies indicate that OmcB from *C. trachomatis* serovar LGV1 functions as an adhesion and is indeed surface exposed (Fadel and Eley, 2007; 2008). OmcA or Omp3 has a molecular of 9-12 kDa. This cysteine rich lipoprotein is anchored to the outer membrane by its lipid moiety with a hydrophilic peptide extending to the periplasm (Everett and Hatch, 1995).

The CRPs of the COMC are present in the outer membrane of the EBs. They do not appear in RBs, only during the differentiation from RB to EB (Newhall, 1987; Sardinia et al., 1988; Everett and Hatch, 1995; Sanchez-Campillo et al., 1999). It has been proposed that disulphide cross-linked polymers of OmcB were the functional equivalent of peptidoglycan forming a disulphide cross-linked network with the

perisplasmic domains of OmcA and other membrane protein like MOMP. This might account for the structural stability of EBs (Hatch et al., 1986; Hackstadt et al., 1985; Everett and Hatch; 1995).

4. 2. 3. Lipopolysaccharide

According to other gram-negative bacteria, the chlamydial outer membrane contains an antigenically important lipopolysaccharide (LPS) with a molecular weight of 10 kDa. Chlamydial LPS harbors a genus-specific epitope composed of a 3-deoxy-D-mannose-oct-2 ulopyranosonic acid (Kdo) trisaccharide with the sequence αKdo-(2→8)-αKdo-(2→4)-αKdo which is surface exposed and highly immunogenic (Brade et al., 1986). The structure is unique to *Chlamydiaceae* because two of the Kdo residues are linked through a 2→8 linkage (Brade et al., 1987).

4. 2. 4. Polymorphic membrane proteins

In 1996, the group of David Longbottom has identified and isolated a group of proteins of approximately 90 kDa present in the outer membrane of *C. abortus* strain S26/3, called the polymorphic membrane proteins or pmps (Longbottom et al., 1996; 1998). Genome sequencing of chlamydial genomes revealed a pmp family unique to *Chlamydiales* that plays an important role in the biology and disease pathogenesis. There are 17 pmp genes identified for *C. Caviae* (Read et al., 2003), 21 for *C. pneumonia* (Kalman et al., 1999), 9 for *C. trachomatis* (Stephens et al., 1998), 12 for *C. felis* (Harley et al., 2007) and 18 for *C. abortus* (Thomson et al., 2005). The pmp genes encode large proteins of 288 to 582 amino acids. These proteins are polymorphic in both amino acids and predicted size. However, they all encode repeats (2-13 copies) of the amino acid sequence GGAI and FXXN in the amino-terminal of the protein (Kalman et al., 1999; Grimwood et al., 2001). They also have the amino acid phenyl-alanine at the amino terminus and most have signal peptides indicating that they are likely membrane proteins. By protein structure analysis, they are predicted to be autotransporters that mediate the translocation of the N terminus to the bacterial surface (Vandahl et al., 2001; Henderson and Lam, 2001). They also contain multiple GGAI motifs, which have been associated in other organisms with adhesion to the host cell (Grimwood and Stephens, 1999).

4. 2. 5. PorB

Genome sequence has revealed a predicted protein CT713 with weak sequence homology to MOMP and a molecular weight of 37 KDa (Stephens et al., 1998). Functional analysis showed pore-forming capabilities when tested in a liposome-swelling assay, but they were different from the porine capabilities of MOMP. Therefore this protein was named PorinB (PorB) because it was the second porin characterized in *Chlamydia* (Kubo and Stephens, 2000). PorB is localized in the outer membrane complex of *Chlamydia* and it is surface accessible but it is present at much lower amounts than MOMP

in the COMC (Kubo and Stephens, 2000). PorB and MOMP are both porins facilitating the diffusion of solutes through the outer membrane. MOMP is a general porin, permitting the diffusion of a wide variety of compounds, like polysaccharides and amino acids including glutamate. In contrast PorB is a more specific porin, transporting dicarboxylic acids such as 2-oxoglutarate in order to complete the tricarboxylic acid (TCA) cycle and providing carbon and energy production intermediates (Iliffe-Lee and McClarty, 2000; Kubo and Stephens, 2001).

4.2.6. Omp85

Omp85 is a conserved outer envelope protein found in a number of Gram-negative bacteria, including *N. gonorrhoeae*, *T. pallidum* and *H. influenza* (Stephens and Lammel, 2001). Omp85 is present in the chlamydial outer membrane complex but no porin activity has been detected so far. Because Omp85 is surface-accesible, antibodies against Omp85 can neutralize chlamydial infection *in vitro*, which make Omp85 a valid candidate for vaccine development (Kubo and Stephens, 2001). Omp85 proteins have been shown to be essential for bacterial cell viability, lipid transport to the outer membrane (Genevrois et al., 2003) and the assembly of outer membrane proteins (Voulhoux et al., 2003).

4.2.7. Heat shock proteins

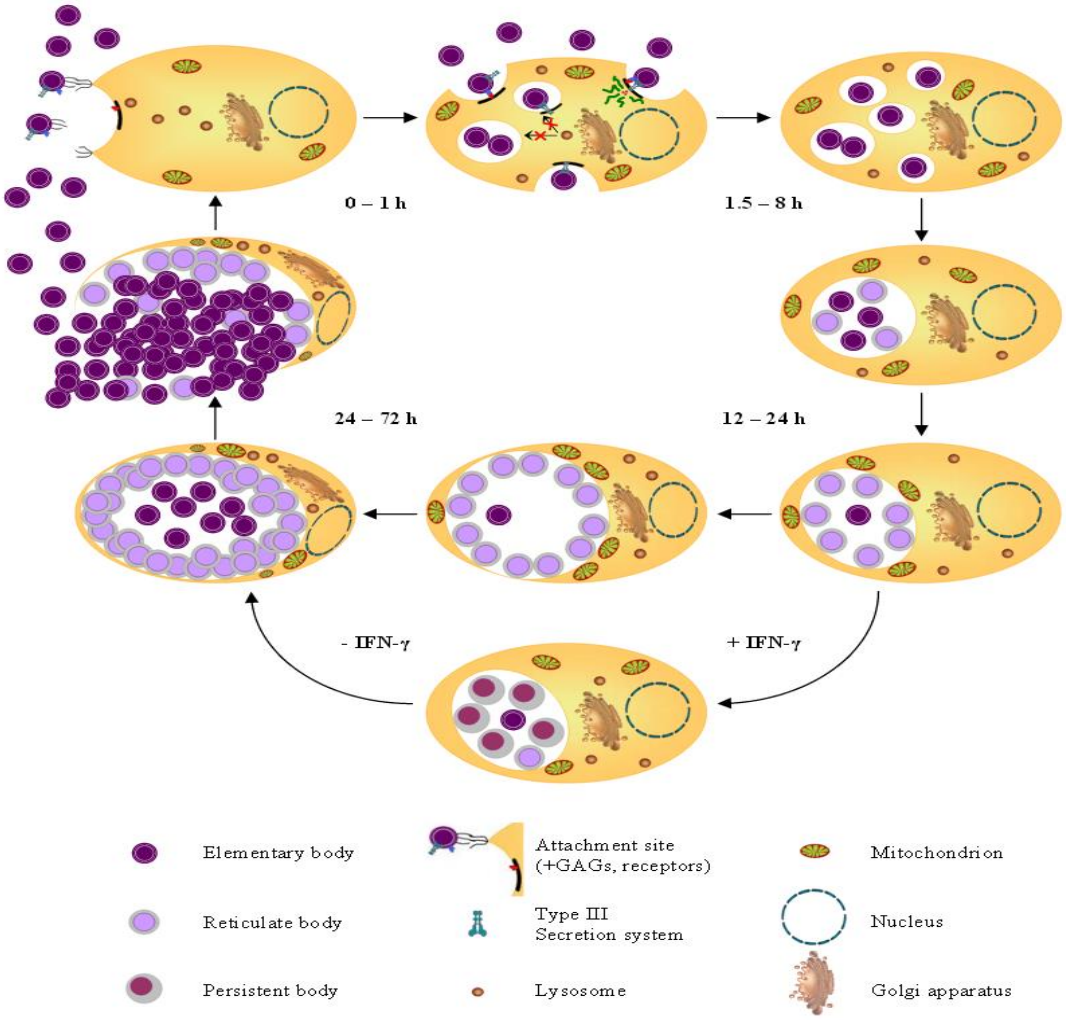
Heat shock proteins (HSP) are highly conserved intracellular proteins. Their amino acid composition has not changed very much throughout evolution and they are expressed in both prokaryotes and eukaryotes. Different chlamydial heat shock proteins have been identified: Hsp10, Hsp60 and Hsp70 (Kornak et al., 1991; Brunham and Peeling, 1994). HSP functions as chaperone proteins concerned with the intracellular folding and refolding, assembly and translocation of proteins. Their expression is increased when cells are exposed to stress like elevated temperature, protein degradation, mechanical stress and chemical stress (Van Eden et al., 1998). In *Chlamydia*, Hsp60 facilitates protein folding (Fink, 1999; Hartl and Hayer-Hartl, 2002; Tsan and Gao, 2009) and antibodies against *C. trachomatis* Hsp60 have been correlated with severe outcome of chlamydial infections in women (Zhong and Brunham, 1992). The chlamydial Hsp70 has been best studied, has a molecular weight of 75 kDa, and prevents the misfolding and aggregation of polypeptides (Fink, 1999; Hartl and Hayer-Hartl, 2002; Tsan and Gao, 2009). This protein shares approximately 50% sequence homology with other species. It is also a potential vaccine candidate as antibodies to the protein can neutralize the organism in cell culture (Danilition et al., 1990). However, the homology between chlamydial and human Hsp is so high that antibodies may crossreact resulting in an auto-immune disease (Capello et al., 2009).

5. Developmental cycle

Chlamydiaceae are obligate intracellular bacteria which means that they can only replicate inside eukaryotic host cells. The chlamydial development cycle is a complex multi-stage process characterized by the entry, the early differentiation, the exponential growth and inclusion expansion, detachment and late differentiation and finally, cell lysis or exocytosis (Figure I-4). *Chlamydiaceae* have a unique biphasic developmental cycle which involves predominantly two alternating morphological forms, the EB and the RB. The EBs have the ability to attach and invade susceptible host cell, whereas the RBs carry out bacterial multiplication within the cells (Stagg, 1998; Abdelrahman and Belland, 2005).

The acute infection starts with the attachment of EBs to the cytoplasmatic membrane of a susceptible host cell inducing its own internalization in membrane bound vacuoles termed inclusions. These inclusions avoid phagosome-lysosome fusion. EBs differentiate into metabolically active RBs and undergo repeated cycles of binary fision leading to secondary differentiation back to EBs. Eventually, EBs and some non-differentiated RBs are released from the host cell through lysis or exocytosis. Under certain conditions like nutrient deprivation, antibiotics or immune factors (IFN-γ), the developmental cycle is disrupted, resulting in the appearance of large, aberrant RBs. This phenomenon is known as persistence, in which no visible growth of the chlamydial organism can be observed (Mpiga and Ravaoarinoro, 2006).

Fig. I-4: Schematic representation of the development cycle of *Chlamydiaceae* (Beeckman, 2009).



5.1 Attachment and internalization

Attachment of EBs to the host cell occurs in a two-steps process. The initial attachment happens via reversible electrostatic interactions of the bacteria with heparin sulfate containing glycosaminoglycans (GAGs) (Zhang and Stephens, 1992). Second, an irreversible, temperature-dependent receptor-ligand binding was demonstrated that induces internalization (Fudyk et al., 2002). So far, the receptor responsible for the binding has yet to be identified. Possible bacterial adhesion candidates include MOMP, OmcB, Hsp70 and PmpD (Raulston et al., 1993, Ting et al., 1995; Wehrl et al., 2004; Hackstadt, 1999). MOMP has also been implicated in the entry of chlamydiae into their host cell. It promotes non-specific electrostatic interactions followed by more specific hydrophobic interactions acting as an adhesion (Su et al., 1990).

Ultrastructural studies have shown that chlamydiae preferentially bind to the host cell near the base of microvilli. This might be advantageous since the membrane regions at the bases of the microvilli are areas of active transport of extracellular material into the cell, possibly facilitating rapid and efficient

entry (Hodinka and Wyrick, 1986; Doughri et al., 1972; Wyrick and Richmond; 1989; Vanrompay et al., 1996; Hodinka et al., 1988). Electron microscopic studies suggest two possible mechanisms for chlamydial internalization. The first one involves a sequential zipper-like microfilament-dependent process of phagocytosis requiring drirect contact between bacterial adhesins and host cell receptors (Byrne and Moulder, 1978). The second one involves uptake into clathrin-coated vesicles by receptor mediated endocytosis (Hodinka et al., 1988; Vanrompay et al., 1996). Following the attachment, the type III secretion system (T3SS) is also activated and effector proteins are secreted into the host cell, including the bacterial protein Tarp (Translocated Actin-Recruiting Phosphoprotein), resulting in actin rearrangements and active uptake of the bacteria by means of an endocytotic vacuole (Abdelrahman and Belland, 2005). *Chlamydiaceae* are able to inject Tarp and other effector proteins because EBs have a preformed T3SS loaded with bacterial effector proteins (Bavoil and Hsia, 1998).

5.2 Inclusion forming and proliferation

After internalization, chlamydiae modify the properties of its inclusion; thereby preventing normal trafficking through the host endocytic pathway, effectively dissociating it from late endosomes and lysosomes. In normal conditions, lysosomes fuses with pathogen-containing phagosomes, releasing acidic hydrolases in the resulting phagolysosomes in order to eradicate the pathogen. *Chlamydiaceae* have developed mechanisms to avoid this phagolysosomal fusion. Many theories have been evoked that attempt to explain this inhibiton and these are summarized in a review by Escalante-Ochoa et al. (1998). However, *in vitro* analyses show that not every inclusion escapes fusion with lysosomes depending on the kind of host cell, chlamydial strain and the conditions (Moulder, 1991). Within a few hours post infection, the inclusions are trafficked to the perinuclear region of the host cell using microtubules (Scidmore et al., 1996). This migration brings the inclusion in close proximity to the Golgi apparatus to acquire endogenously synthesized spinghomyelin and cholesterol (Hackstadt et al., 1995; Rockey et al., 1996; Wolf and Hackstadt, 2001). These processes occur early in the developmental cycle and are necessary for successful replication of *Chlamydiaceae* within a host cell.

Meanwhile, by 8 hours post infection, primary differentiation from EBs into metabolically active RBs takes place. Subsequently, these RBs migrate to the periphery of the inclusion and start replication by binary fission resulting in exponential growth of the pathogen and increase in inclusion size. At the beginning of the reproductive stage, the chlamydial inclusion is closely associated with mitochondriae, facilitating the transfer of ADP and ATP to the bacteria. Remarkably, this association is only seen in infections with *C. psittaci* (Matsumoto et al., 1991). The T3SS remains active in RBs and continues to secrete effectors into the host cytoplasm. An important group of proteins secreted by the T3SS are the Inc proteins which insert into the inclusion membrane and are thought to interact with host proteins.

They share a common hydrophobicity motif that span the inclusion membrane (Rockey et al., 2002). Interestingly, the inclusion must be actively modified otherwise the chlamydiae will be degraded.

Following the bacterial replication, RBs will re-differentiate into EBs, termed secondary differentiation. From 24 hours post infection, asynchronous replication occurs resulting in RBs that continue to replicate and RBs that will differentiate into infective EBs (Dunn and Valdivia, 2010). The signal for this secondary differentiation is unknown, but speculative mechanisms have been proposed that the dissociation of dividing RBs from the inclusion membrane could be a trigger. The removal of the T3SS from the inner surface of the inclusion membrane has been suggested as a critical event in this process (Bavoil et al., 2000). This hypothetical mechanism could also explain the asynchronous nature of secondary differentiation.

5.3 Exit from the host cell

After secondary differentiation, the newly formed EBs and some non-differentiated RBs will exit the host cell at 24 to 72h post infection through several mechanisms: 1) host cell lysis (Rockey et al., 1996; Campbell et al., 1989), 2) exocytosis (Todd and Caldwell, 1985), 3) caspase-independent apoptosis (Perfettini et al., 2002) and 4) extrusion (Hybiske and Stephens, 2007). In this way, EBs will infect neighbouring cells, thereby spreading the bacterium, and consequently the disease, and enabling further proliferation of the bacteria in the host. Because *Chlamydiaceae* reside within an inclusion, it needs to traverse two membranes to escape the host cell. Lysis of the host cell is a destructive mode of release leading to rupture of the inclusion and cellular membrane, resulting in the death of the host cell. Upon lysis, EBs can diffuse outward and infect new cells. In contrast, extrusion represented a packaged release process in which a portion of the chlamydial inclusion was released by membrane protrusion. This process left the original cell and residual inclusion intact. But the consequence of extrusion-mediated release is less clear. It is possible that rupture and release of EBs occur after they have moved to a safer location, away from preexisting local immune responses. Alternatively, these extrusions could also be engulfed by macrophages, in which secondary infection and dissemination in the host are facilitated (Hybiske and Stephens, 2007).

5.4 Persistence

Chlamydiaceae have the ability to interrupt the developmental cycle, resulting in a persistent infection. A persistence infection is determined as a long-term relationship between *Chlamydia* and their host cell in which these bacteria remain in a viable but culture-negative state. *In vitro*, persistence can be induced by iron deficiency (Raulston, 1997), monocyte infection (Koehler et al., 1997), continuous culture (Kutlin et al., 2001), penicillin treatment (Matsumoto and Manire, 1970), phage infection (Hsia

et al., 2000), amino acid starvation (Coles et al., 1993) and IFN-γ exposure (Beatty et al., 1993). The *in vitro* persistence systems are characterized by altered Chlamydial growth, describing enlarged and pleomorphic RBs that neither undergo binary fission, nor differentiate to EBs, but nevertheless continue to replicate their chromosomes. These RBs are called aberrant RBs.

6. Type III secretion system in Chlamydiales

6.1. Introduction

The type III secretion system (T3SS) has been described in a wide variety of Gram-negative pathogens including *Yersinia*, *Salmonella*, *Shigella*, *Escherichia*, *Pseudomonas*, *Bordetella*, *Burkholderia*, *Chlamydiaceae* and a number of plant pathogens (Cornelis and Van Gijsegem, 2000; Hueck, 1998). This system functions as a kind of 'molecular syringe', enabling Gram-negative bacteria to translocate virulence-related proteins into the cytoplasm of host cells (Hueck, 1998). The injected proteins subvert the functioning of the host cell or destroy its communication, favoring the entry or survival of the invading bacteria.

There are seven major classes of protein secretion systems, which can be divided into Sec-dependent and Sec-independent systems. The Sec-dependent secretion systems deliver proteins into the periplasmic space from which the proteins are transported across the outer membrane. Sec-independent secretion systems, as the T3SS, deliver proteins directly from the bacterial cytosol across the bacterial membranes (Thanassi et al., 2000; Economou et al., 2006).

Decades before the first visualization of a T3SS in *Salmonella*, electron microscopic observations revealed the presence of many hexagonally organized surface projections arranged regularly with a center to center spacing of approximately 50 nm of both EBs and RBs of different chlamydial species. These structures have a rotational symmetry corresponding to a 9-subunit composition (Matsumoto, 1973; 1982; Matsumoto et al., 1976), which were later assumed to be the T3SS of the bacteria (Fields et al., 2003).

Genome sequencing showed that *Chlamydiaceae* possesses most of the genes for a functional T3SS (Stephens et al., 1998), which are scattered throughout chlamydial genomes in four main clusters (Fields and Hackstadt, 2006) arranged into multiple operons (Hefty and Stephens, 2007). The molecular G+C content of each chlamydial T3S cluster is similar to the roughly 40% level found in the remainder of respective genomes (Stephens et al., 1998). This is in contrast to T3S genes of other Gram-negative bacteria which are clustered in pathogenicity islands (PAI) on a plasmid or the bacterial chromosome, suggesting that they have been acquired by horizontal gene transfer (Mecsas and Strauss, 1996). Furthermore, the nucleotide content of these T3SS coding sequences are often denoted by a low G+C

ratio (Tampakaki et al., 2004). However, some of the chlamydial T3S clusters lack gene arrangements consistent with horizontal gene transfer or PAIs and are flanked by tRNAs and insertion elements. These observations have provided the basis for hypothesis stating that the T3SS of *Chlamydiaceae* represent an ancestral or primordial system (Stephens et al., 1998; Kim, 2001), which is functionally adapted to the developmental cycle of *Chlamydiaceae* and the need of these bacteria to survive host defenses on both sides of the eukaryotic plasma membrane.

6. 2. Composition of the chlamydial T3SS

The basal structure of a T3SS consists of a basal body and an external hollow needle through which effector molecules are transported. The basal body connects both the inner and outer membrane and forms the base for the needle structure. At the tip of the needle, adaptor proteins are assembled which are involved in host cell sensing. Upon contact with the target cell, a pore is formed in the host cell membrane by translocon proteins. In that way, effector molecules can be transported from the bacteria into the host cell cytoplasm.

The T3SS is regulated in two important ways. Firstly, the assembly of the T3SS is tightly controlled at transcriptionally, translationally and post-translationally level in a yet undefined manner. Secondly, the secretion of effector molecules is controlled by a blocking plug, the SctW protein. Activation of the T3SS results in the secretion of a this blocking plug along with the translocon proteins. The blocking plug is able to block the T3S channel in order to control the secretion of effectors proteins (Lara-Tejero et al., 2011; Osborne and Coombes, 2011).

The channel of the needle is too small (2-3 nm) to allow folded proteins to pass through the needle. Therefore, effector molecules need to be (partially) unfolded for translocation (Stebbins and Galan, 2003; Parsot et al., 2003). Generally, chaperones aid the non-covalent folding or unfolding and the assembly or the disassembly of other macromolecular structures. They play an important role in the T3S function via their interaction with effector and translocator proteins. T3SS chaperones can be separated into five classes based on their substrate specificity and structural features. Class I chaperones assist the effector proteins, while chaperones of Class II interact with the translocator proteins. Chaperones of the related bacterial flagellar apparatus belong to Class III. Class IV has only one member, CesA which accompanies the EspA filament protein of enteropathogenic *E. Coli* (Yip et al., 2005). Yersinia YscE and its Pseudomonas homolog PscE with their co-chaperones YscG and PscG group together in Class V (Wilharm et al., 2007). Only Class I and Class II will be highlighted as they are well characterized. Class I chaperones interact with effector proteins, directing them to the ATP-ase, which unfolds effector proteins and detach the chaperone (Galan, 2008). As the hydrophobic translocator proteins possess toxic properties for the bacteria, they are protected by class II

chaperones (Menard et al., 1994; Wattiau et al., 1994; Neyt and Cornelis, 1999). The concentration of free chaperones is an indication if the pore of the T3SS is made (Page and Parsot, 2002).

The discovery of different genes encoding proteins involved in T3SS led to the exception that *Chlamydiae* possess a T3SS (Hsia et al., 1997). The chlamydial T3S model was described by Beeckman and Vanrompay (Beeckman and Vanrompay, 2010), classifying the T3SS into several components: the exporter, the basal body, the secreton, the needle and the translocon (Figure I-5).

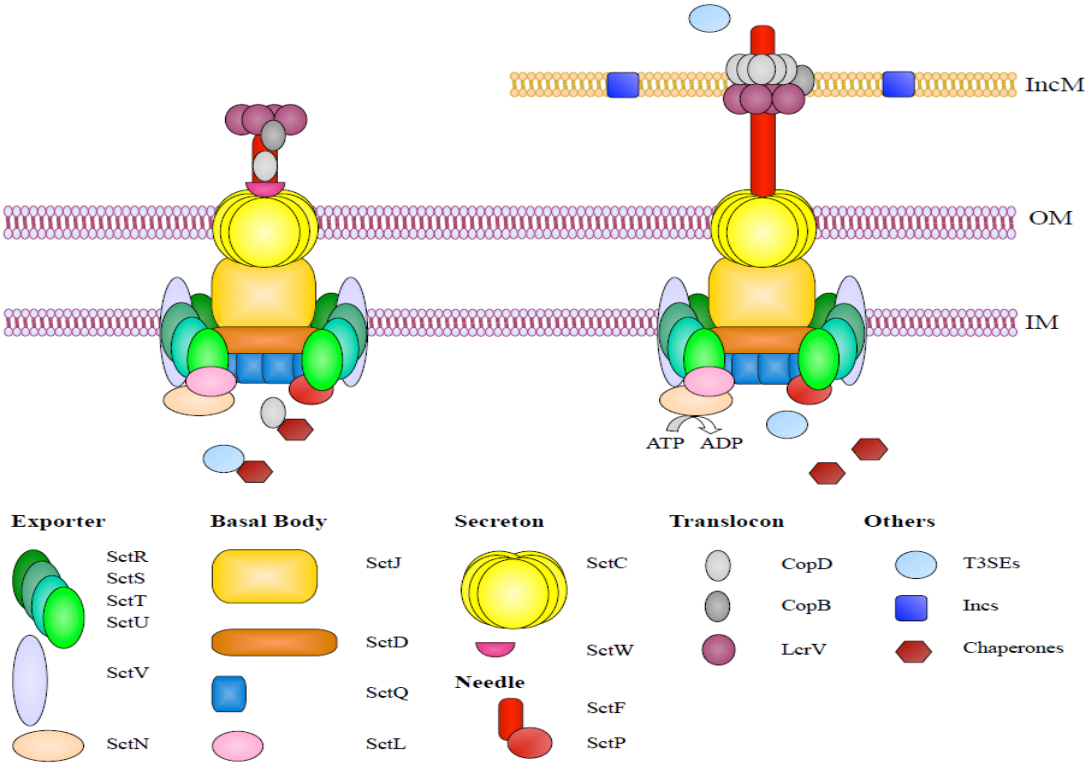


Fig. I-5: Schematic representation of Type III secretion system in *Chlamydiaceae* as suggested by Beeckman and Vanrompay (2010). On the left side is an inactive T3SS shown, while the right side represents a T3SS after activation. Legend: IM, bacterial inner membrane; OM, bacterial outer membrane; IncM, Inclusion membrane.

The exporter surrounds the basal body and consists of several proteins (SctR, SctS, SctT, SctU, SctV and SctN) located in the inner membrane at its cytoplasmic face. The exporter is responsible to mediate active transport of effector proteins to the periplasmic space. The proteins SctR, SctS, SctT and SctU are membrane proteins. The protein SctV forms a channel though which effector proteins can cross the inner membrane (IM). The protein SctN uses ATP to give energy to the T3SS and it also plays a role in the interaction with effector proteins through ATP-dependent unfolding and chaperone release prior to translocation (Akeda and Galan, 2005; Sorg and Cornelis, 2009). The proteins SctJ, SctD, SctQ and SctL form the basal body which is needed for the correct anchoring of the needle complex. The secreton consists of the proteins SctC and SctW and is involved in the transport of effector molecules

across the outer membrane. SctD and SctC are connected by the lipoprotein SctJ that bridges the outer and the inner membranes (Allaoui et al., 1992; Hueck, 1998). The protein StcQ is responsible for shuttling chaperone/effector complexes to the protein SctN (Stone et al., 2008) for unfolding and chaperone release. The proteins SctF and SctP make up the hollow needle, where T3SS proteins can travel to cross the membranes. As mentioned above, the translocon pore is built into the host cell membrane upon host cell contact sensed by a multimeric protein complex of LcrV at the needle tip, which are also known as adaptor proteins (Blocker et al., 2008). CopB and CopD form a heterodimeric translocon in the host cell membrane that enables the needle to dock and subsequently deliver effector molecules in the cytoplasm of the host cell.

As opposed to the conserved feature of the T3SS, effector proteins are highly divergent in sequence and function between bacterial species. Most of these effector molecules mimic the host cell proteins to utilize and manipulate the host cell. There are able to interact with the host without significant harm, to modify the cellular targets to alter their activity and to evade and escape the host cell immune response. Effector proteins can act in concordance with other effector proteins to result in a highly complex field of interactions (Galan 2009; Shames and Finlay, 2010, Brodsky and Medzhitov, 2009). The secreted T3S effector proteins and their possible function are summarized in table I-2.

Table. I-2: Overview of chlamydial T3S effector proteins and their possible function (Beeckman and Vanrompay, 2010; Peters et al., 2007; Betts et al., 2009; Betts-Hampikian and Fields, 2010).

Effector protein	Possible function
IncA	Inclusion membrane protein
IncB	Inclusion membrane protein
IncG	Inclusion membrane protein
Tarp	Invasion, recruitment and nucleation of actin at the site of entry
CADD	Modulation of host cell apoptosis
Mip	Induction of cytokine response
Cap1	$CD8^+$ T-cell antigen
Pkn5	Ser/Thr protein kinase activity
Ca037	Ruler protein involved in determination of needle length
CT147	Circumvent lysosomal fusion
CT668	Unknown
CT694	Interaction with human AHNAK
CT874	Interaction with human GCIP

6. 2. *Role of T3SS in the chlamydial lifecycle*

The T3SS plays a pitoval role in the chlamydial developmental cycle. It modifies the intravacuolar environment to favor the different stage of the chlamydial lifecycle.

During adhesion and entry in the host cell, the effector molecule Tarp (translocated actin-recruiting phosphoprotein) is translocated within minutes following attachment and induces actin recruitment to invade the host cell. During infection, *Chlamydiaceae* have developed mechanisms to avoid the phagolysosomal fusion, which is most likely accompanied by an active modification of the inclusion membrane by chlamydial Incs (Hackstadt et al., 1997; Wyrick, 2000). Furthermore, the inclusion of *Chlamydiaceae* mimicks the phospholipid composition of the host cell. In this way, there are able to intercept nutrient and essential molecules from the eukaryotic trafficking pathways of the host cell like sphingomyelin, glycerophospholipids and other lipids (Hatch and Mc Clarty, 1998). Later on, physical detachment of RBs from the inclusion membrane provokes T3S downregulation with upregulation of the SctW protein expression, which is the signal for the differentiation from RBs into EBs (Hackstadt et al., 1997; Bavoil and Hsia, 1998; Bavoil et al., 2000).

7. Avian chlamydiosis

7.1. Classification of avian strains

All known avian strains belong to the species *Chlamydia psittaci* within the order of *Chlamydiales*. *C. psittaci* can be further classified with serovar-specific monoclonal antibodies against serovar-specific epitopes of the MOMP into six avian serovars (serovar A to F) along with two mammalian serovars (WC and M56) (Andersen, 1991; Vanrompay et al., 1993; Vanrompay et al., 1997b). Progress in the field of molecular techniques has led to the sequencing of the *ompA* gene -encodes the major outer membrane protein MOMP-, which allow to classify avian *C. psittaci* strains into genotypes. These genotypes are largely congruent with the known serovars, which can be characterized by sequencing (Everett et al., 1999), genotype-specific real-time PCR (Geens et al., 2005) or micro-arrays (Sachse et al., 2008). However, sequencing of the *ompA* gene has revealed a new genotype, namely genotype E/B. As genotype E/B reacts with the serovar-specific monoclonal antibodies E and B, it is impossible to distinguish this genotype with monoclonal antibodies.

Table. I-3: Overview of *C. psittaci* genotypes

Reference strain	Genotype	Host
VS1	A	Psittacine birds
CP3	B	Pigeons
GD	C	Ducks/geese
NJ1	D	Turkeys
MN	E	Pigeons/turkeys
VS225	F	Psittacine birds
WS/RT_E30	E/B	Ducks
M56	M56	Muskrat/hare
WC	WC	Cattle

The currently known genotypes tend to be relatively host-specific. Genotype A has been shown to infect psittacine birds, whereas genotype B is most prevalent in pigeons. Genotype C has been primarily isolated from waterfowl such as ducks and geese and genotype D has the highest prevalence in turkeys. Genotype E has the most diverse host range infecting pigeons, ratites, ducks and turkeys. Genotype F has been detected in in psittacine birds and turkeys and genotype E/B has been so far mainly isolated from ducks. WC and M56 has been shown to occur in Wolfsen cattle and muskrats, respectively. All genotypes should be considered as a zoonotic risk as they are readily transmissible to humans.

7.2. Epidemiology

From 1938, it became clear that *C. psittaci* not only infects psitaccine birds but also other bird species like poultry such as chickens, turkeys, ducks and geese but also wild birds (Vanrompay et al., 1995a). In the early 1950s, *C. psittaci* could be isolated from turkeys and humans due to a severe respiratory outbreak in the turkey industry in the US (Meyer, 1967). Psittacosis outbreaks were also reported in the European poultry industries in the 1990s (Ryll et al., 1994; Vanrompay et al., 1997; Sting et al., 2006). Recent publications show considerable evidence that *C. psittaci* is nearly endemic in the poultry industry in Belgium and other European countries (Sting et al., 2006; Van Loock et al., 2005; Verminnen et al., 2008; Dickx et al., 2010; Yin et al., 2013a; Lagae et al., 2013). Nowadays, farm outbreaks seem to be associated with mild respiratory signs and low mortality. However, it has been recently reported that chlamydiosis increases in people working with poultry (Laroucau et al., 2008; Laroucau et al., 2009, Van Droogenbroeck et al., 2009; Dickx et al., 2010, Lagae et al., 2014). At this moment, *C. psittaci* has been found in at least 465 bird species, spanning 30 different bird orders (Kaleta and Taday, 2003).

7.3. Transmission

Transmission of *C. psittaci* from one bird to another susceptible bird occurs by inhalation of aerosols created from respiratory tract exudates, eye and nostril secretions and faecal material containing the organism (Burkhart and Page, 1971; Takahashi et al., 1988; Cole, 1990). Shedding occurs intermittently and can be activated through stress e.g. sudden change of environment, nutritional deficiencies, overcrowding, transport, breeding programs, handling and egg laying (Fudge, 1996). The excretion period depends on the immune status of the host, the virulence of the strain and the infection dose, but shedding may occur for several months (Gerlach, 1999). Another route to become infected is by blood-sucking ectoparasites, like mites, lice and flies (Shewen, 1980; Page, 1975), or less common, through bites or wounds (Longbottom and Coulter, 2003). Vertical transmission of *C. psittaci* through the egg is demonstrated in chickens, turkeys, ducks, parakeets, seagulls and snow geese (Wittenbrink et al., 1993; Vanrompay et al., 1995a; Lublin et al., 1996).

7.4. Pathogenesis, clinical signs and macroscopic lesions

After inhalation of *C. psittaci*–containing aerosols, primary replication starts in epithelial cells of the upper respiratory tract (nose, trachea, bronchi). Later on, the bacteria can be found in epithelial cells and macrophages of the lower respiratory tract (bronchiole, lungs and airsacs). Subsequently, *C. psittaci* can be demonstrated in plasma- and blood monocytes resulting in a systemic infection affecting various organs like the liver, spleen, pericardium, gut and bone marrow (Vanrompay et al., 1995a). The incubation period varies from three days to several weeks (Page, 1959) and depends on the immune status of the host, the virulence of the strain and the infection dose. Common symptoms of the infection include sneezing, coughing, ocular and nasal discharge, sinusitis, rhinitis, dyspnoea, diarrhea, polyuria, dehydration, watery droppings, fever, anorexia and lethargy (Grimes and Wyrick, 1991; Longbottom and Coulter, 2003; Vanrompay et al., 1995a). Depending on the age and immune status of the host, stress condition, virulence of the strain and the infection dose, symptoms can vary from inapparent to severe. Looking at the macroscopic lesions during necropsy, the severeness of the infection can be observed. Generally, congestion of conjunctivae, conchae and trachea is present. Furthermore, thickened airsacs, grey inflammatory spots in the lungs, fibrinous pericarditis and enlarged liver and spleen can be observed (Grimes and Wyrick, 1991; Longbottom and Coulter, 2003).

7.5. Diagnosis

Several techniques are available to detect *C. psittaci* in clinical samples (Harkinezhad, 2008). These techniques include: 1) cell culture for the isolation of *C. psittaci*, 2) Enzyme-linked immunosorbent assay (ELISA) for chlamydial antigen/antibody detection, 3) immunohistochemical staining of histological sections, which is based on the presence of the *Chlamydiaceae* family-specific LPS, 4) DNA-based detection methods (PCR, real-time PCR and micro-array).

7.6. Treatment

To treat avian chlamydiosis, the antibiotic tetracycline and its derivates are usually used, although alternatives like quinolones (enrofloxacin) and macrolides (azithromycin) can also be administered (Vanrompay et al., 1995a). At this moment, antibiotics are the only way to manage with *C. psittaci* infections (Van Droogenbroeck, 2010). However, regular and abundant antibiotic use can create resistant pathogens, which can lead to huge animal losses but also human disease due to *C. psittaci*. This is already the case for the related organism *C. suis* causing conjunctivitis and reproductive failure in swine, as publications have reported tetracycline resistance and horizontal gene transfer (Dugan et al., 2007; Dugan et al., 2004; Schautteet et al., 2010).

7.7. Prophylaxis

To cope with the problems of antibiotic use, prophylaxis become more and more important. Preventive measures must be taken to prevent or reduce transmission of *C. psittaci* between birds. Firstly, proper and regular cleaning and disinfection of the cages is very important, as *C. psittaci* is able to survive for 30 days in faeces. Detergents like isopropyl alcohol, lysol, quaternary ammonium compounds, chlorophenols and household bleach are very effective in the battle against *C. psittaci* (Longbottom and Coulter, 2003; Smith et al., 2005). Secondly, the development of an effective vaccine would be of great importance to prevent avian chlamydiosis. However, at this moment, no commercial vaccine is available (see section 9). Thirdly, the natural anti-microbial protein ovotransferrin (OvoTF) has shown *in vitro* and *in vivo* to have an anti-chlamyial effect (Beeckman et al., 2007; Van Droogenbroeck et al., 2008).

8. Human psittacosis

8.1. Public health significance

Human psittacosis is an infectious disease and therefore designated as a reportable disease. However, the incidence of this disease is underestimated because not all infections with *C. psittaci* cause pneumonia and therefore remain unnoticed. Furthermore, psittacosis is challenging to diagnose and control. Some birds may be carriers and look healthy but actually they are shedding the *C. psittaci* organism intermittently. Persons at risk include bird fanciers, employees in poultry slaughtering, veterinarians, laboratory workers, workers in avian quarantine stations, wildlife rehabilitators, zoo workers and pet shop workers (Harkinezhad et al., 2007; Vanrompay et al., 2007).

8.2. Transmission

Humans can become infected with *C. psittaci* by inhalation of aerosols created from respiratory and eye secretions, dried faeces and urine of infected birds. Other sources of exposure include handling of the plumage and tissues of infected birds, a bite from an infected bird and in rare cases mouth-to-beak contact (Beeckman and Vanrompay, 2009; Smith et al., 2005). Even brief exposures can lead to symptomatic infections as well as unrelated activities like gardening or law mowing (Telfer et al., 2005; Williams et al., 1998). Person-to-person transmission of the disease is rare (Hughes et al., 1997; Williams et al., 1998; Ito et al., 2002).

8.3. Clinical presentation

Human psittacosis varies in severity from a mild flu-like illness to severe pneumonia. Generally, the incubation period is 5 to 14 days, but periods up to 1 month are also possible. Symptoms include abrupt onset of fever, chills, headache, malaise and myalgia. Usually, a non-productive cough is developed, which can be accompanied with breathing difficulty and chest tightness. Primarily, human psittacosis affects the respiratory system but the bacteria may spread throughout the body affecting different organs like the liver, the heart, the gastro-intestinal tract and the brain. When the gastro-intestinal tract is infected, symptoms like vomiting, abdominal pain and diarrhea are common. In rare cases, complications like encephalitis (Carr-Locke and Mair, 1976), hepatitis (Samra et al., 1991), myocarditis, endocarditis (Levison et al., 1971), adult respiratory distress syndrome (ARDS) (Yilmazlar et al., 2000), arthritis (Gonski and Chan, 2009), keratoconjuntivitis (Dean et al., 1995), ocular adnexa lymphoma (Chanudet et al., 2006; Ferreri et al., 2004) and multiple organ failure (Heddema et al., 2006) may occur. The outcome of the disease is variable and can be fatal in untreated patients (Kovacova et al., 2007).

8.4. Diagnosis

A correct anamnesis of the patient's medical history, professional and free time occupations is a first step to link flu-like symptoms to human psittacosis. If the clinical representation is in concordance with human psittacosis, several laboratory tests are available to confirm the disease. First, serological tests like microimmunofluorescence (MIF) test, complement fixation test (CFT) and ELISA show cross-reactivity with other chlamydial species, which makes it difficult to distinguish *C. psittaci*, *C. trachomatis* and *C. pneumoniae* antibodies in humans. Furthermore, those tests generate both false negative and false positive results (Verminnen et al., 2008). Second, molecular techniques for the detection of *C. psittaci* DNA were developed. Several nested PCRs based on detecting of the 16S rRNA and *ompA* gene are now routinely being used (Sachse and Hotzel, 2003; Van Loock et al., 2005b). Moreover, *C. psittaci* genotyping could be performed using a genotyping real-time PCR (Geens et al., 2005) or a genotyping micro-array (Sachse et al., 2008; Sachse et al., 2009). Third, the causative agent could be isolated from respiratory secretions on cell cultures. Different cell lines such as Buffalo Green Monkey (BGM), McCoy, HeLa and African Green Monkey (Vero) are being used, but BGM cells showed to be the most sensitive artificial host (Vanrompay et al., 1992). Disadvantages from this technique are that the bacteria need 3 to 6 days to grow sufficiently and only a limited of laboratories perform this technique because a biosafety level 3 (BSL3) facility is required.

8.5. Treatment

As in birds, human psittacosis is preferentially treated with antibiotics with tetracyclines such as doxycycline or tetracyclinehydrochloride, unless this drug is contra-indicated (pregnant women, children < 8 years, allergic reaction). Macrolides such as erythromycin and azithromycin can also be used as treatment (Senn et al., 2005). Treatment with antibiotics can easily resolve chlamydial infections but resistance can occur through horizontal gene transfer as this is already the case for *C.suis* which has acquired tetracycline resistance (Dugan et al., 2004; Dugan et al., 2007). As the use of tetracyclines is widespread in pet birds and poultry, tetracycline resistance can also occur among *C. psittaci* strains. Moreover, resistance can also take place through point mutations which can alter the expression and functionality of the bacteria (Dreses-Werringloer et al., 2003; Roshick et al., 2000). Furthermore, antibiotics can induce subclinical persistent infections evolving to chronic disease when antibiotic administration is stopped (Hogan et al., 2004).

9. Vaccination

Until today, vaccination is still the most effective way to prevent or even eradicate infectious diseases in humans and animals (Bowersock and Martin, 1999; Oshop et al., 2002). The ultimate goal of a vaccine is to stimulate the immune system to develop sufficient amounts of specific antibodies, cytotoxic T cells and memory T- and –B cells against a specific pathogen. The need for an efficient vaccine against *Chlamydiae* is huge, as there are some disadvantages linked to the use of antibiotics to treat chlamydial infections. First, pathogens can become resistant to antibiotic treatment because of the regular and abundant use. Second, chlamydial infections are often asymptomatic and so only those with clinical signs are treated which is not the best way to control the infection (Stagg, 1998). Third, a persistent infection can be induced due to antibiotic treatment. Fourth, the use of antibiotics does not always stop the shedding of the organism which is the case for *C. abortion* at lambing or oestrus (Longbottom and Coulter, 2003). Vaccination would be the best approach to control and prevent chlamydial infections. Today, no commercial vaccine against chlamydiosis/psittacosis exists. To make an effective vaccine it is important to identify those antigens that elicit a protective immune response, to understand the complex immune mechanisms that occur during infection and to develop effective methods for vaccine delivery (Longbottom, 2003).

9.1. Whole organism vaccines (first generation)

In the 1950s and 1960s, vaccine studies focused on the use of inactivated or live, attenuated whole organisms in order to control both animal and human infections. The first vaccines that were used against *Chlamydiaceae* were live vaccines, which are attenuated or living chlamydial organisms.

Generally, developing an attenuated vaccine is done by a number of passages of the pathogen in different types of cell cultures. Due to the several passages, one or more mutations occur, resulting in a non-virulent attenuated strain of the pathogen. A major advantage is that this live vaccine very closely reproduces a normal infection, resulting in a natural stimulus of the immune system (both cellular and humoral) against all protective antigens. But live vaccines can revert to its virulent form and cause disease or persistent infection, especially in immunosuppressed individuals. Because of the unsafety of live vaccines, killed or inactivated micro-organisms were used. These are preparations of the wild-type pathogenic micro-organism that have been rendered non-pathogenic using heat or chemical treatment. As the micro-organism cannot replicate, the use of these vaccines are safe but a large number of inactivated pathogens are required to stimulate the immune system, which stresses the need to revaccinate and to use adjuvantia. Furthermore, only a humoral immune response can be induced. As *Chlamydiaceae* has an intracellular life cycle, a strong cell-mediated immune response is needed for the clearance of a chlamydial infection, making killed or attenuated pathogens less suitable as vaccine against *chlamydiaceae* (Van Drunen Little-van den Hurk et al., 2000; Longbottom and Livingstone, 2006). Another major disadvantage of whole organism vaccines is that they contain both immunogenic and non-immunogenic components and immune answer against non-immunogenic components is irrelevant to prevent infection and it even has leads to a reduction of the immune response against immunogenic components (Shams, 2005). Undesirable components such as endotoxins can result in pathological damage in the host (Wegener et al., 1998). In general, whole organism vaccines seems to be more successful in controlling animal infections than human infections. The most promising attempts with first generation vaccines have been made to control ovine enzootic abortion (OEA) caused by *C. abortion* in sheep. This formalin-inactivated whole organism vaccine was commercialized and successfully applied for 20 years but the re-occurrence of the disease in vaccinated animals has led to the withdrawal of the vaccine in 1992 (Littlejohn et al., 1952; McEwen et al., 1951; McEwen and Foggie , 1954; Longbottom and Livingstone, 2006). Nowadays, three inactivated vaccines and one killed vaccine against OEA are commercially available in the EU and the USA. Furthermore, the only other commercially available vaccines are against *C. felis*, which include two inactivated- and five live attenuated vaccines. These vaccines reduce acute infection but do not always prevent shedding of the pathogen or re-infection (Wills et al., 1987).

9.2. Subunit vaccines (second generation)

The second generation– or subunit vaccines contain a part of the pathogen, which are one or more defined antigenic determinants playing an important role in the protective immunity. This can be antigens or parts of antigens, represented in several forms including purified proteins, recombinant proteins and synthetic peptides (Hess et al., 2000). Because the material in a subunit vaccine is not

able to replicate, the risk of adverse reactions is very low and safe for use in immunosuppressed persons. Despite their safety, subunit vaccines are less successful at inducing long lasting immunity against disease and need to be administered repeatedly along with adjuvantia. The main development of vaccines against *Chlamydiacea* is focused on MOMP, a structurally and immunologically dominant protein (Caldwell et al., 1981). Initial experiments with MOMP purified from SDS-PAGE failed to induce protection against *C. trachomatis* in monkeys (Taylor et al., 1988). This failure was probably due to the denaturation of protective conformational epitopes on the surface of the bacteria during sample preparation for gel electrophoresis. Other attempts using detergent extracted COMC preparations showed that MOMP offer protection in sheep (Tan et al., 1990), guinea pigs (Batteiger et al., 1993) and mice (Sandbulte et al., 1996; Pal et al., 1997). However, purifying MOMP and COMC preparations are difficult and expensive because of the problematic bulk growth of *Chlamydiaceae*. Therefore, recombinant proteins could offer a solution to this concern.

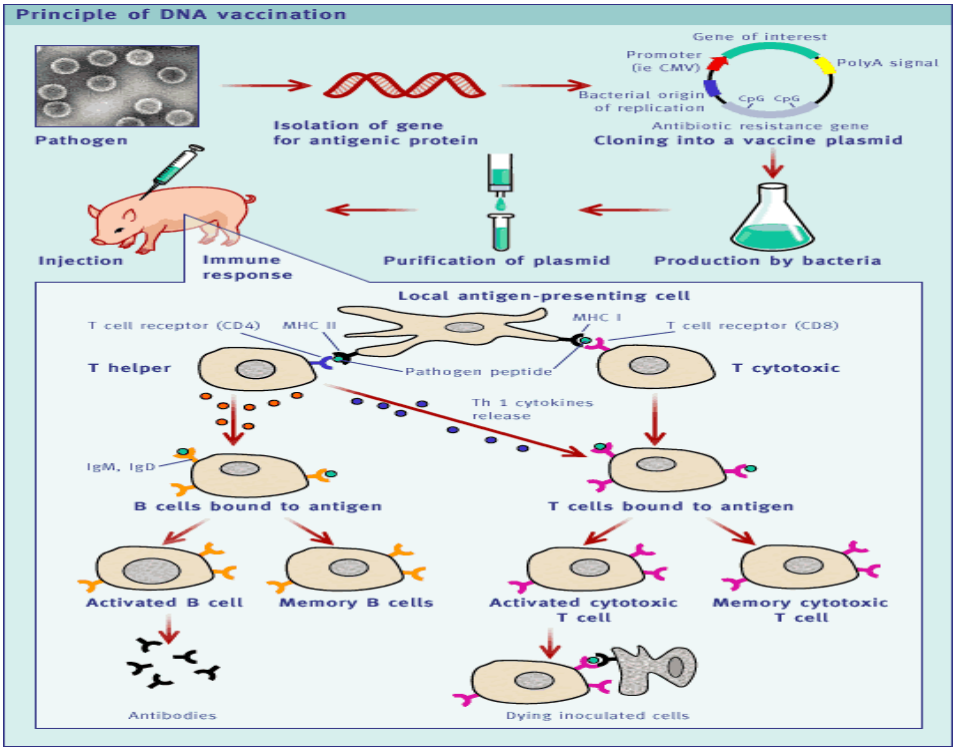
Because of the DNA technology, developing recombinant protein vaccines will be easier and cheaper to prepare than the MOMP and COMC preparations which needed large-scale production of *Chlamydiaceae*. However, the expression of the porin MOMP is often toxic in prokaryotic expression systems and it is also difficult to produce the native form of rMOMP with intact conformationally relevant epitopes (Longbottom, 2003). Moreover, MOMP is also glycosylated (Escalante-Ochoa et al., 1998; Swanson and Kuo, 1991) and it is possible that export and assembly of MOMP is incompatible with other proteins of other gram-negative bacteria as the composition of the cell wall is different (Manning and Stewart, 1993). Therefore, it has been showed that the production of rMOMP is easier in eukaryotic cells, such as COS7 cells (Vanrompay et al., 1998). A study of Verminnen et al. (2005) testing a rMOMP vaccine with and without 1α,25-dihydroxyvitamin D_3 in turkeys showed partial protection. Furthermore, a rMOMP vaccination study of Tuffrey et al. (1992) did not result in a complete protection against *C. trachomatis* in mice. As most vaccination strategies with subunit vaccines are focused on rMOMP, the results are not satisfying.

9.3. DNA vaccines (third generation)

DNA vaccination could be the new strategy to protect humans and animals against *Chlamydiaceae*. A DNA vaccine consists of a plasmid encoding the gene(s) of antigenic component(s) of interest. Injection of this plasmid can result in the intracellular production of the chosen antigen(s) in the host cell, inducing a protective immune response. By mimicking the natural way of infection, both humoral and cellular immune responses may be elicited which is ideal to combat intracellular pathogens like *Chlamydiaceae*. The principle of DNA vaccination is shown in figure I-6. First, the gene of interest is selected, isolated and inserted into a mammalian expression vector system. Once constructed, the

DNA vaccine is transformed into bacteria, where bacterial growth generates multiple plasmid copies. Afterwards, the plasmid DNA is purified from the bacteria and ready to be delivered in animals (Dufour, 2001; Oshop et al., 2002). This DNA vaccine can be injected into the skin or muscle of the host, which causes the host cell to produce the introduced gene. This protein is endogenously produced and intracellularly processed into small antigenic peptides by host proteases. Those peptides enter the lumen of the endoplasmatic reticulum and are presented by MHC class I molecules. Subsequent $CD8^+$ cytotoxic T lymphocytes (CTL) are stimulated which inhibit pathogens through both cytolysis of infected cells and noncytolosis mechanisms such as cytokine production (Encke et al., 1999). Exogenous antigens can also be presented in association with MHC class II molecules, stimulating $CD4^+$ T cells.

Fig. I-6: Principle of DNA vaccination (Dufour, 2001).



DNA immunization has many advantages when compared with other vaccination strategies (Watts and Kennedy, 1999). DNA vaccines induce the expression of antigens that resemble native epitopes more closely than standard vaccines do since live attenuated and killed vaccines are often altered in their protein structure and antigenicity. Even post-translation modifications are similar as those present during infection (Babiuk et al., 2003). Moreover, the plasmid vector can encode several antigens that can be delivered to the host in a single dose. Furthermore, rapid and large-scale production is possible and the DNA can be easily purified, resulting in lower costs to develop an effective DNA vaccine (Dufour, 2001; Longbottom and Livingstone, 2006). DNA vaccines are also temperature stable, which makes storage and transport much easier (Ertl and Xiang, 1996). Because of the endogenous

production of the antigen(s), a humoral as well as a cellular immune response is evoked and even a more balanced Th1/Th2 like immune response, which is very important in the battle against intracellular pathogens (Ulmer et al., 1998). DNA vaccines elicit also an immune response in neonates and in the presence of maternal antibodies (van Drunen Littlel-van den Hurk et al., 2000; Van Loock et al., 2004). This advantage is of very great interest to combat *C. psittaci* in the poultry, as newborn chicks have a poorly developed immune system (Oshop et al., 2002) and high maternal antibody titers against *C. psittaci* have been found in the offspring of most hens. Unfortunately, there are also some disadvantages linked to the usage of DNA vaccines. As plasmid vectors often contain nucleic acid sequences from oncogenic viruses, the possibility exist that DNA integrate in the host chromosome. Although, this has not been proven yet and it is believed that the spontaneous mutations frequency is lower (Martin et al., 1999). There is also a possibility that after injection of bacterial DNA, an immune response is evoked generating antibodies against DNA. These antibodies might cross-react with host DNA, potentially causing auto-immune diseases in the host (Watts and Kennedy, 1999). Furthermore, tolerance can be induced to the antigen (protein) produced. Non-proteins based antigens such as bacterial lipopolysaccharides and lipids showed not to be suitable in DNA vaccination strategies (Watts and Kennedy, 1999).

Several studies has investigated the protection of DNA vaccines against *Chlamydiaceae*. In a murine respiratory model, DNA vaccination with the *ompA* gene induced a protective immune response (Zhang et al., 1997) and vaccination with *ompA* DNA followed by a booster with rMOMP protected mice against a swine *C. abortus* infection (Zhang et al., 2009). However, protection could not be induced in mice against *C. pneumonia* using DNA vectors expressing the cytoplasmic heat shock protein 60 (Hsp60) or the outer membrane proteins, MOMP and *Omp2* (Penttila et al., 2000). The same result was obtained after vaccination with the *dnaK* gene of *C. abortus* in mice (Hechard et al., 2003a; Hechard et al., 2003b). Although, a protective immune response could be induced against *C. psittaci* using a MOMP-based DNA vaccine in turkeys even if maternal antibodies were present (Van Loock et al., 2004; Vanrompay et al., 1999a; Vanrompay et al., 1999b; Vanrompay et al., 2001; Verminnen et al., 2005; Harkinezhad et al., 2009b; Verminnen et al., 2010). Nowadays, three DNA vaccines for veterinary use have been approved. There is one against infectious hematopoietic necrosis in Atlantic salmon (Meeusen et al., 2007), one to protect horses against the West-Nile virus (Powell, 2004) and one against melanoma in dogs (Bergman et al., 2006).

9.4. Adjuvants

An effective vaccine must stimulate the two arms of the immune system, the innate and the adaptive immunity and generate a long-term protection against infection. However, most vaccines developed

today include small components of the pathogen. To generate a strong, long-lasting protective immune response, molecules such as adjuvants are needed to enhance the specific immune response (Shams, 2005; Wilson-Welder et al., 2009). The word 'adjuvant' is derived from the Latin word 'adjuvare', which means to help or to enhance (Sanders et al., 2005; Rajput et al., 2007). Different adjuvants like alum-based adjuvants, Freund's adjuvants, immunostimulating complexes (ISCOMs), CpG motifs and bacterial toxins can be used, depending on the antigen, the species, the route of administration and potential side-effects. An ideal adjuvant should be biodegradable, cheap, promote an effective immune response and have a long shelf life (Allison and Byars, 1990). Generally, adjuvants can be used for various purposes: 1) boost the immune system against certain antigens or subvert the immune response to particular cell types of the immune system, 2) reduce the amount of antigen or the number of immunization, 3) act as an antigen delivery system for better uptake of the antigens by the mucosa, and 4) to ameliorate the efficacy of vaccines in immunosuppressed people, the elderly and newborns (McElrath, 1995; Cox and Coulter, 1997).

10. Immune response

Humans and animals are constantly being exposed to infectious agents and in most cases these infections can be resisted. The immune system is composed of the innate or non-specific immune system and the adaptive or the specific immune system, both combatting pathogens. *Chlamydia* is one of the many pathogens, able to invade and cause infection in humans and animals. *Chlamydia* possesses a unique developmental cycle, alternating between two morphologic forms – the extracellular, metabolically inactive and infectious EB and the metabolically inactive and infectious RB that divides by binary fission within the inclusion. Consequently, the elicited immune response and its regulation during infection that contribute to host resistance or susceptibility have not been fully elucidated yet. However, understanding the evoked immune response by *Chlamydiae* is an important issue to understand the pathogenesis of acute and persistent infections but also for developing targets for effective vaccination and therapeutic strategies for infection.

10.1. Innate immune response

The very first line of defense against *Chlamydiae* includes physical and chemical barriers such as the skin, mucous membranes, hairs and cilia, gastric juice, vaginal secretions, urine, tears, sweat, saliva, cerumen, tight junctions of the cells and commensals. Once the outer line of defense is breached, innate immune effectors provide the next line of defense.

10. 1. 1. Pattern recognition receptors

Pattern recognition receptors (PRRs) are on the front line of inducing innate immune response and are capable of sensing conserved microbial molecules, referred as pattern associated molecular patterns (PAMPs), found on the bacterial surface or inside the pathogen, as well as damage-associated molecular patterns (DAMPs). PRRs are located intracellulary or on the plasma membrane of immune cells and also on epithelial cells. The different known PRRs can be seen as a network that lead to the activation of the same downstream components in response to an infection.

10.1.1.1 Toll-like receptors

Toll-like receptors (TLRs) are pattern recognition receptors that recognize highly conserved structural motifs expressed by microbial pathogens like lipid, carbohydrate, peptide and nucleic acid structures. Stimulation of the TLRs initiates signaling cascades leading to the activation of transcription factors, such as AP-1, NF-κB and interferon regulatory factors (IRFs), resulting in a variety of cellular responses like the production of cytokines, interferons (IFNs) and effector cytokines that direct the adaptive immune response. *Chlamydiaceae* are classified as gram-negative bacteria, suggesting that LPS plays an important role in driving the innate immune response against all types of chlamydial infections. However, a study of Tsutsumi-Ishii et al. (2007) showed that *Chlamydiaceae* LPS has low binding affinities for its recognition molecules such as CD14 and LBP (LPS Binding Protein) and exhibit weak biological activities like cytokine production against host immune cells including monocytes, thereby contributing to the chronic (persistent) inflammatory reactions during infection. However, during a *C. pneumoniae* infection, chlamydial LPS and Hsp60 – both ligands of TLR4 – mediates macrophage foam cell formation and DC maturation (Chen et al., 2008; Bulut et al., 2009). Furthermore, TLR4 also recognizes *C. trachomatis* LPS (Prebeck et al., 2003), but a study of Darville et al. showed that TLR2 is a more important mediator in the innate immune response to Chlamydial infection. Macrophages from TLR2 knock-out (KO) mice produced less TNF-α and IL-6 in response to an active *C. trachomatis* infection. In contrast, macrophages of TLR4 KO mice seemed to produce more TNF-α and IL-6 when compared with normal infected mice, suggesting that binding of *Chlamydiae* by TLR4 may down modulate the signaling of other TLRs (Darville et al., 2003). Furthermore, TLR2 seem to be essential for bacterial clearance and survival during *C. pneumonia* infection *in vivo* (Rodriguez et al., 2006; Beckett et al., 2012), because *C. pneumoniae* do activate NF-κB in a TLR2 dependent manner, leading to cytokine production in dendritic cells and macrophages (Prebeck et al., 2001). However, the chlamydial pathogen associated molecular pattern (PAMP) that binds with TLR2 still need to be identified (O'Connell et al., 2006). In conclusion, it is likely that both TLR2 and TLR4 are necessary to induce a cytokine and chemokine response and a protective host defense against chlamydial infections *in vivo*,

with TLR2 playing the greater role. In general, the intracellular TLR9 recognizes unmethylated CpG sequences present in bacterial genomes or viral DNA. Han et al. (2004) observed upregulation of mRNA TLR9 in dendritic cells during intranasal chlamydial infection. Most probably, TLR9 has a role in modulating the immune response towards a Th1 cellular response (Han et al., 2004).

The myeloid differentiation primary response gene 88 (MyD88) is an universal adaptor protein in the downstream signaling pathway of different TLRs (TLR2, TLR4, TLR5, TLR7-9) and the IL-1 receptor family. A study of Naiki et al. (2005) demonstrated that after infection with *C. pneumonia* MyD88-deficient mice could not upregulate pro-inflammatory cytokines and chemokines, showed a delayed recruitment of $CD8^+$ and $CD4^+$ T cells to the place of infection and the bacteria could not be removed from their lungs. Furthermore, those mice developed a severe, chronic lung inflammation with increased mortality, indicating that MyD88 is essential for the initiation of an effective host immune response against the battle of *C. pneumonia*e and it bridges innate immunity to Th1 responses. However, *C. pneumoniae* was also able to induce TRIF activation, which is a crucial adaptor protein in the MyD88-independent signaling pathway. TRIF- as well as MyD88 activation mediates foam cell formation in macrophages during *C. pneumoniae* infection (Chen et al., 2008).

In summary, both TLR2 and TLR4 signaling via MyD88 control chlamydial infections through the early production of cytokines and chemokines. Moreover, activation of TLR-2 and TLR-4 influence their own expression as well as those of the cytokine receptors, leading to a strong and well-orchestrated immune response.

10.1.1.2 NOD-like receptors

The nucleotide-binding oligomerization receptors or NOD-like receptors (NLRs) are intracellular receptors of PAMPs, and orchestrate an inflammatory response, autophagy or cell death. The well described NOD-like receptors are NOD1 and NOD2 and recognize distinct motifs of peptidoglycan (PGN), an essential component of the bacterial cell wall, which is covalently closed , net-like polymer in which glycan strands made of alternating N-acetylglucosamine and N-acetylmuramic acid residues are cross-linked by peptides. NOD1 recognizes D-γ-glutamyl-meso-DAP dipeptide (iE-DAP), which is found in PGN of all Gram-negative bacteria and certain Gram-positive bacteria. NOD2 senses intracellular muramyl dipeptide (MDP), found in almost all bacteria (Franchi et al., 2009, Chen et al., 2009).

However, *Chlamydiae* lacks detectable amounts of PGN, but these bacteria do possess PGN synthesis and show sensitivity against Penicillin, which is a PGN inhibitor. This paradox is known as the *Chlamydiae* anomaly. Moreover, several studies have reported a significant role for NOD1 induction of inflammation during infection with *C. muridarum*, *C. pneumoniae* and *C. trachomatis* (Opitz et al., 2005; Whitmarsh and Davis, 1996). The endogenous IL-8 production elicited by *C. trachomatis* in HeLa

cells is dependent upon NOD1 signaling through its signaling partner receptor-interacting protein 2 (RIP2) (Buchholz and Stephens, 2008). Furthermore, a reduced IFN-β expression after infection with *C. muridarum* was observed in HeLa cells treated with NOD1 siRNA (Prantner et al., 2010). Shimada et al. (2009) investigated the importance of the NOD/RIP2 pathway in host responses after *C. pneumoniae* infection in mice. $NOD1^{-/-}$, $NOD2^{-/-}$ and $RIP2^{-/-}$ mice showed a delayed bacterial clearance compared to wild-type (WT) mice, suggesting that *C. pneumoniae* is sensed by both intracellular receptors. In conclusion, the NOD/RIP2 signaling pathway plays a significant role in intracellular recognition of the different *Chlamydiae* species, in the innate host immune response and finally in the clearance of the bacteria and the survival of the host. However, there must be a perfect coordinated and sequential activation of both TLRs and NODs signaling pathways to evoke a well-balanced host immune defense against *Chlamydiaceae*. Probably upon chlamydial infection, TLRs are necessary for the initial activation, wheras NODs are responsible for the intracellulary prolonged activation of target cells as *Chlamydiae* is an intracellular bacteria. These data above plus the sensitivity to penicillin suggest that *Chlamydiae* do bear PGN or a PGN-like structure on its cell wall despite the lack of biochemical evidence. It is possible that the amount of PGN in *Chlamydiae* is so low that it is below the detection level. Moreover, *Chlamydiae* do switch between the infectious EB and the non-infectious RB, making a biochemical detection of PGN difficult (Hammerschlag, 2002). Penicillin is able to alter the morphology of EBs and to stop the devolpmental cycle, indicating that penicillin act on newly synthetized structures during maturation from EB to RB (Matsumoto and Manire, 1970).

10.1.1.3 Inflammasomes

Danger associated molecular patterns (DAMPs), also known as alarmins are molecules released by stressed cells undergoing necrosis that act as endogenous danger signals to promote and exacerbate the inflammatory response. Several researchers believe that DAMPs released by injured cells are necessary for the host to distinguish pathogenic microorganisms from commensal and other non-pathogenic bacteria (Matzinger, 2002; Seong and Matzinger, 2004). Some NLRs sense those DAMP molecules and form large cytoplasmatic complexes, called inflammasomes. The assembly of inflammasomes leads to the production of active caspase-1, which is responsible for the transformation of pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18. To generate the immature forms of IL-1β and IL-18, NF-κB activation is necessary resulting from signaling such as TLRs. It has been reported that *C. pneumoniae* induce IL-1β secretion in macrophages through the direct activation of the NLRP3/ASC inflammasome and subsequently caspase-1 in coordination with TLR2 and MyD88 which generate pro-IL-1β (Shimada et al., 2011; He et al., 2010). Moreover, caspase-1 deficient mice showed a higher bacterial burden in the lungs and an increased mortality during *C. pneumonia*e infection and IL-1β administration diminished those effects, especially during the early stages of the

infection (Shimada et al., 2011). It is clear that caspase-1 dependent IL-1β is necessary to initiate a proper host immune response against *Chlamydiae*, whereas NLRP3 inflammasome plays an important role in this process. However, as mentioned before, the molecular mechanism for NLRP3 activation still need to be unraveled. NLRP3 recognize danger-associated molecules like reactive oxygen species (ROS) (TSchopp and Schroder, 2010), lysosomal damage (Hornung and Latz, 2010) and cytosolic K^+ efflux (Pétrilli et al., 2007), rather than pathogen-associated molecules. As *chlamydiae* damage the mitochondria in macrophages (Shimada et al., 2011), the mtDNA in the cytosol can promote inflammasome assembly (Shimada et al., 2012), leading to the production of IL-1β. This mitochondrial damage in macrophages causes apoptosis, whereas IL-1β is secreted before cell death, acting as an alarming danger signal. However, it has been demonstrated that *Chlamydiae* also inhibit apoptosis in favor of his developmental cycle (Fan et al., 1998; Fischer et al., 2001; Geng et al., 2000). In conclusion, these data suggest that *Chlamydiae* struggle with his host cell the macrophage, 1) resulting in apoptosis and subsequently preventing further replication of the pathogen or 2) resulting in inflammasome assembly and propagation of pro-inflammation via IL-1β. However, it is also known that activation of inflammasomes also induce cell death known as pyroptosis via activation of caspase-1. Thus, besides secretion of pro-inflammatory cytokines such as IL-1β and IL-18, activation of caspase-1 induces pore formation in the plasma membrane leading to cell rupture and consequent release of cytosolic contents (Martinin et al., 2002; Fink and Cookson, 2006). During *C. trachomatis* infection, the role of inflammasome and subsequently caspase-1 activation is still controversial. Caspase-$1^{-/-}$ mouse fibroblasts are resistant to *C. trachomatis* infection (Jorgensen et al., 2011), whereas caspase-1 deficient mice showed a reduced inflammatory damage at the place of infection (Cheng et al., 2008). Caspase-1 dependent cell death is regulated by the *C. trachomatis* T3SS-dependent effector protein chlamydial protease-like activity factor (CPAF). However, the inhibition of pyroptosis by CPAF is not yet fully understood. It has been suggested that CPAF may function as a proteolyse regulating the pool of T3SS effectors in the host cytosol and avoiding accumulation of PAMPs which can be sensed by intracellular PRRs (Jorgensen et al., 2011).

10. 1. 2. Cytokines/chemokines

After recognition of different PAMPs and DAMPs by several PRRs, a pro- and anti-inflammatory response is mediated by cytokines and chemokines. All families of PRRs activate three major signaling pathways: the nuclear factor (NF)-κB, the mitogen-activated protein kinases (MAPKs) and the interferon-regulatory factors (IRFs) leading to the induction of inflammatory and antimicrobial mediators. The aim of this response is to control the invading pathogen without generating excessive tissue damage and provide an adequate stimulus for the adaptive immune immune system.

Chlamydiaeceae infect epithelial cells as well as cells of the immune system like monocytes and macrophages. The induced set of cytokines and chemokines and the concentration is dependent on the cell type, the route of infection, the dose and the chlamydia species and/or the serovar used. Infection with *C. trachomatis* of epithelial cells causes the induction of IL-8, IL1α, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 (Rasmussen et al., 1997). It must be noticed that depending on the tissue, several cytokines and chemokines are continuously expressed to maintain an innate immune surveillance. Furthermore, *Chlamydiales* can infect immune cells, although not with the same efficiency like epithelial cells. The replication of *Chlamydiaceae* happens in a lower extent in immune cells. This growth inhibition is probably due to TNF-α, as treatment with antibodies against TNF-α causes bacterial growth (Haranaga et al., 2003). Macrophages infected with *C. trachomatis* release TNF-α, which induces apoptosis of T-cells together with other components. Moreover, the factors released during apotosis of T-cells induce the production of transforming growth factor-β (TGF-β) which is an immunosuppressing cytokine, creating an ideal environment for chlamydial persistence (Jendro et al., 2002). This controlled apoptosis prevent bacterial clearance but provide also enough time to complete the developmental cycle or induce persistence. Moreover, a study of Beeckman et al. (2009) showed that *C. psittaci* infected macrophages produce high levels of the anti-inflammatory cytokine IL-10, which can induce deactivation of macrophages and suppression of the NF-κB pathway. The cytokine TNF-α is also produced by macrophages of aborted ovine placentas (Buxton et al., 2002) and elevated levels has also been found in tears of humans suffering from trachoma (Conway et al., 1997). This cytokine can induce an imbalance at mucosal surfaces where Th-2-type cytokines such as TGF-β and IL-10 tend to predominate (Mowat, 2003). This imbalance can be detrimental to the host, particularly if a persistent infection develops that drives an inflammatory loop. However, the cytokines TGF-β and IL-10 are anti-inflammatory cytokines that can counteract the protective anti-chlamydial Th-1-type response, certainly at mucosal sites where those cytokines predominate, resulting in a failure of the host to clear the infection and therefore develop chronic disease (Stephens, 2003). The protective Th-1-type response against *Chlamydiaceae* is mediated by IFN-γ, IL-6, IL-1β, IL-8, IL-12 and GM-CSF. The cytokine IL-8 is an activator and chemoattractant of neutrophils associated with immunopathology (Mukaida et al., 1998; Sansonetti et al., 1999). During a C. trachomatis infection, IL-8 is highly induced and dependent upon NOD1 PRR signaling (Bucholz and Stephens, 2008). The cytokines IL-6 and IL-1β positively influence each other and are implicated in the acute phase response maintaining the inflammation localized to the site of infection, clearing cell debris and attracting other immune (Kaiser et al., 2004; Schneider et al., 2001). Elevation of these cytokines was observed after infection of HD11 cells (chicken monocytes/macrophages) with *C. psittaci* (Beeckman et al., 2010), but also after infection with *C. trachomatis* (Rasmussen et al., 1997). Another major inflammatory cytokines

produced during chlamydial infection is IFN-γ. The cytokine IFN-γ induces antibody isotype switching towards the production of IgG2a, which can be used as a marker of a Th1 response (Snapper and Paul, 1987). IFN-γ restricts *Chlamydiae* growth by inducing the host enzyme indoleamine 2,3-dioxygenase (IDO) that degrades intracellular pools of tryptophan (*Chlamydiae* is auxotrophic for tryptophan). Besides, IFN-γ downregulate the transferrin receptor on the surface of infected cells, which may also limit chlamydial growth by limiting the intracellular stores of iron (Byrd and Horwitz, 1993). In this way the developmental cycle of *Chlamydiae* is delayed, so RBs persist longer which might result in persistent unapparent infection and also contribute to immunopathogensis by promoting inflammatory damage and fibrosis (Rottenberg et al., 2002). In addition, IFN-γ promote the phagocytosis of macrophages, resulting in engulfing and destruction of extracellular EBs (Zhong and de la Maza, 1988). Furthermore, IFN-γ triggers the macrophage to release inflammatory mediators which promotes the destruction of *Chlamydiae* and induces fibroblast proliferation and thereby enhances the synthesis of collagen (Beatty et al., 1993). It has been shown that early production of IL-6 prevent IFN-γ like responses in cells. Given that cells almost directly produce IL-6 following infection with *Chlamydiae* and that IFN-γ is a key cytokine for exerting host immune control, this could explain the pattern of chronic disease seen in many cases of chlamydial infection.

10.2. Adaptive immune response

The adaptive or the acquired immune system is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogen growth. Activation of the adaptive immune system to a particular pathogen leads to the creating of immunological memory. The adaptive immune response consists out of the cell-mediated immunity and the humoral immunity.

10. 2. 1. Cell-mediated immunity

As *Chlamydiae* is an intracellular pathogen, it has been suggested that the cell-mediated immunity would be more effective than the humoral immunity at conferring protection against disease. It has been demonstrated in T cell-depletion and –adoptive transfer experiments that T cells are essential for the protection against chlamydial infections (Ramsey and Rank, 1991; Rank et al., 1985). Moreover, it has been observed that both $CD4^+$ and $CD8^+$ T cell subsets are present at the place of infection (Morrison and Morrison, 2000; Penttila et al., 1998; Rank et al., 2000; Van Voorhis et al., 1996). There is evidence that the presence of $CD4^+$ T cells is more important in the resolution of infection and orchestrating the activation of other protective immune components (Rank, 2000). B cell knockout mice treated with only anti-CD4 antibodies were not able to resolve a chlamydial infection, indicating that $CD4^+$ T cells are essential to generate protective immunity in mice both in primary infection and reinfection (Su et al., 1997; Morrison et al., 2000). However, there is more and more evidence that

$CD8^+$ T cells are also important in the contribution to protection. For instance, depletion of $CD8^+$ T cells in mice abrogates protection upon challenge with *C. psittaci*. Similarly, increased bacterial load and disease severity was observed in the absence of $CD8^+$ T cells during a primary and secondary infection with *C. pneumonia* (Penttila et al., 1999; Rottenberg et al., 1999). Nevertheless, $CD4^+$ T cells are needed for the induction and preservation of a functional $CD8^+$ T cell response and in their absence, both $CD4^+$ and $CD8^+$ T cell effector functions are impaired. The protective cell mediated immune response is strongly associated with the production of IFN-γ produced by T cells (Igietseme, 1996). As mentioned above, IFN-γ reduces the growth of *chlamydiae* by altering the cell metabolism and reducing essential nutrients such as iron and tryptophan.

10. 2. 2. Humoral immunity

The exact role of antibodies in protection against chlamydial infections is difficult to assess, as it is dependent on the site of infection, the chlamydial challenge dose, whether it is a primary or secondary infection and whether the infection is acute or persistent. A study of Ramsey et al. (1988) showed that B cell-deficient mice were able to resolve a chlamydial genital infection equivalent to controls and they were even immune to reinfection, indicating that antibodies are not necessary during primary infection or to obtain protective immunity. Of interest, when mice recovered from chlamydial genital infection were treated with antibodies against CD4 and CD8 and then reinfected, the infection was prolonged in mice treated with anti-CD4-antibodies. However, the infection did not resolve in B cell-deficient mice treated with anti-CD-antibodies, indicating that antibodies and $CD4^+$ T cells are necessary to resolve a secondary infection (Morrison et al., 2000). B cells seem to have a potential role in building up the memory response. There are different mechanisms by which antibodies can resolve a chlamydial infection. The first mechanism is neutralization, such that secreting antibodies bind with EBs and prevents them from attachment to the host cells. In this way, the developmental cycle is abrogated (Howard, 1975; Su and Caldwell, 1991, Peeling et al., 1984; Peterson et al., 1991; Cotter et al., 1995). As *Chlamydiaceae* is an intracellular organism, antibodies can only bind when the bacteria is released from the cell or during reinfection of pre-exposed humans/animals. The second mechanism is that antibodies can opsonize EBs and enhance uptake, ingestion and destruction of chlamydial particles by phagocytes (Moore et al., 2002). The third mechanism is the antibody-dependent cellular cytotoxicity (ADCC), which is the destruction of *Chlamydiae* infected host cells by antibodies (Moore et al., 2002).

Chapter II

Emerging Chlamydia psittaci infections in chickens and examination of transmission to humans

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Abstract

Chlamydia psittaci and atypical *Chlamydiaceae* infections are (re)-emerging in chickens. We therefore examined the prevalence of *C. psittaci*, atypical *Chlamydiaceae* and their zoonotic transmission on 19 Belgian chicken farms. Atypical *chlamydiaceae* were not detected in chickens but 18 of 19 farms were positive for *C. psittaci* by culture and PCR. *C. psittaci ompA* genotypes A and D were discovered. None of the examined humans (n= 31) was infected with atypical *Chlamydiaceae*, but 29 (93.5%) of them were positive for *C. psittaci* by culture and PCR. Genotypes A, D and a mixed infection with genotypes C and D were found. Humans (n = 2) working in the *C. psittaci* negative farm never had respiratory complaints, while 25 out of 29 (86.2%) positive farmers, reported yearly medical complaints potentially related to psittacosis. Four of them currently experienced respiratory disease and one of them was being treated with antibiotics. Four farmers (12.5%) mentioned that they had pneumonia after starting to keep chickens. Occupational physicians should be aware of emerging *Chlamydiaceae* infections in chickens.

1. Introduction

The species *Chlamydia psittaci* (*C. psittaci*) causes respiratory disease in birds including symptoms such as rhinitis, conjunctivitis, nasal discharge, dyspnoea, diarrhoea, polyuria, anorexia, lethargy and dullness (Vanrompay *et al.,* 1995). *C. psittaci* is a well-known zoonotic agent causing psittacosis or parrot-fever in humans. During the last 3 decades, psittacosis outbreaks were reported in the United States (Grimes & Wyrick, 1991; Newman *et al.,* 1992), China (Ni *et al.,* 1996), India (Chahota *et al.,* 2000), Australia (Tiong *et al.,* 2007) and European poultry industries (Laroucau *et al.,* 2009; Ryll *et al.,* 1994; Sting *et al.,* 2006; Van Loock *et al.,* 2005; Vanrompay *et al.,* 1997). Zoonotic transfer occurs through inhalation of contaminated aerosols originated from feathers, fecal material and respiratory tract exudates. Handling the plumage, carcasses and tissues of infected birds and in rare cases, mouth-to-beak contact or biting also carry a zoonotic risk (Beeckman & Vanrompay, 2009). Psittacosis in humans may vary from unapparent to fatal in untreated patients (Kovacova *et al.,* 2006). Symptoms include high fever, chills, headache, myalgia, non-productive coughing and difficult breathing (Beeckman & Vanrompay, 2009).

C. psittaci infections mostly occur on turkey or duck farms. However, *C. psittaci* infections are emerging in European and Asian chickens. Recently, Dickx *et al.,* (2010) examined Belgian broiler breeder, broiler and layer farms by a *C. psittaci* recombinant major outer-membrane protein (MOMP) -based antibody ELISA (Verminnen *et al.,* 2006) and found 98%, 95%, and 95% seropositive layers, broilers, and broiler breeders, respectively. Moreover, they demonstrated *C. psittaci* genotype D in the air of chicken hatching chambers and in slaughtered Belgian and French broilers. Zoonotic transmission to hatchery and abattoir employees did occur (Dickx *et al.,* 2010; Dickx *et al.,* 2011), albeit without severe clinical consequences. Recently, Yin *et al*., (2012), proved Hill's-Evans' postulates for *C. psittaci* genotype B and D strains isolated from Belgian and French broilers.

Larouceau *et al.,* (2009) detected a new atypical chlamydial agent in chickens. The atypical chicken *Chlamydiaceae* (ACC) caused apparently no disease in infected chickens, but the detection of ACC coincided with three cases of atypical pneumonia in individuals working in a French poultry abattoir. In 2012, ACC have been detected in Australian, German, Greek, Croatian, Slovenian and Chinese chicken flocks (Robertson *et al.,* 2010; Zocevic *et al.,* 2012). Importantly, ACC are not detected with *C. psittaci*-specific molecular tools, rendering the need for an ACC-specific PCR. The zoonotic potential and the exact taxonomic status of ACC have yet to be defined.

The aim of the current study was to examine the presence of *C. psittaci* and ACC on Belgian chicken farms, as well as their zoonotic transmission to farmers.

2. Material and Methods

2.1. Study concept

We investigated the presence of *C. psittaci* and ACC, as well as their zoonotic transmission, on 19 Belgian chicken farms: seven broiler breeder (1600 to 50,000 animals), seven broiler (200 to 150,000 animals) and five layer (7000 to 22,000 animals) farms from four difference geographical regions (Antwerp, East-Flanders, West-Flanders and Limburg). Only one out of 19 farms kept additional birds species (ducks and geese). The study was conducted in the summer of 2012. Participating poultry farms were randomly recruited by phone. A sampling package was brought to each poultry farm and sampling was performed immediately. The package contained a questionnaire designed to asses information on: 1) the farmers' professional and nonprofessional activities, smoking habits, general health status, use of medication, influenza vaccination, allergies, clinical signs potentially related to psittacosis, 2) the chicken breed, hatchery, housing, feeding, health status, medication, mortality and 3) the presence of other animals on the farm. The package also contained rayon-tipped aluminium shafted swabs (Copan, Fiers, Kuurne, Belgium) for pharyngeal sampling of ten randomly selected chickens and the farmers (a maximum of two per farm). Sampling of the chickens was performed by one of the researchers. In the meantime, humans sampled themselves at home after informed consent. Swabs for culture contained 2 ml chlamydia transport medium (Vanrompay *et al.,* 1992) while those for PCR contained 2ml DNA stabilization buffer (Roche, Brussels, Belgium). Packages were transported on ice and stored at -80°C until use.

2.2. Chlamydia psittaci culture

Culture was performed using Buffalo Green Monkey (BGM) cells and the organism was identified using the IMAGEN direct immunofluorescence assay (Oxoid, United Kingdom) at 6 days post-inoculation (Vanrompay *et al.,* 1994). *C. psittaci* positive cells were monitored using a CX31 fluorescence microscope with a x600 magnification (Eclipse TE2000-E, Nikon, Japan) and represented by a score ranging from 0 to 5 (Table II-1).

Table II-1: *C. psittaci* culture scores

Score	*Interpretation
0	Negative (no EB, no IPC)
1	1-5 EBs
2	6-15 EBs
3	15-25 EBs and 1-5 IPCs
4	25-100 EBs and 6-15 IPCs
5	1-10 EBs/field and 1-5 IPCs/field

*EB = elementary body, IPC = inclusion positive cell

2.3. *Chlamydia psittaci* genotyping and PCR detection of atypical *Chlamydiaceae*

DNA extraction of swabs was performed as described by Wilson *et al*. (1996). Briefly, specimens were centrifuged (13,000 x g), suspended in 198 µl STD buffer (0.01 M Tris-HCl [pH 8.3], 0.05 M KCl, 0.0025 M MgCl2.6H20, 0.5% Tween20) and 2 µl proteinase K (20 mg/ml stock solution; Sigma Chemical Co.). The specimens were incubated at 56°C for one hour and subsequently heated at 100°C for 10 min.

A *C. psittaci*-specific nested PCR with internal inhibition control was used (Van Loock et al., 2005b). Outer membrane protein A (*ompA*) genotyping was performed by a *C. psittaci* genotype-specific real-time PCR (Geens *et al.,* 2005). The latter PCR distinguishes genotypes A to F and E/B using genotype-specific primers, genotype-specific probes and competitor oligonucleotides. Samples from chickens and humans were also examined for atypical chicken *Chlamydiaceae* by use of a recently developed 16S rRNA-based ACC-specific real-time PCR (Zocevic *et al.,* 2013).

2.4. Statistics

Potential zoonotic risk factors were statistically examined using SPSS (Inc., Chicago, Illinois, US). Logistic regression was used to search for non-exposure-related risk factors for *Chlamydiaceae* culture and PCR positivity. The model contained data on the information acquired from the questionnaire.

3. Results

3.1 Chlamydia psittaci and atypical Chlamydiaceae in chickens

Nineteen out of 32 (59%) chicken farms contacted participated, resulting in samples from 190 chickens (10 per farm) and 31 humans (a maximum of two per farm).

Atypical chicken *Chlamydiaceae* were not detected. Eighteen out of 19 farms (94.7%) were positive for *C. psittaci* by both culture and nested PCR (Table II-2). The percentage of culture-positive chickens per farm varied from 60 -100%. *C. psittaci* genotype D was present in 17/18 (94.4%) of positive farms, while a genotype A infection was discovered in 1/18 positive farms (Table V-3). Thus, *C. psittaci* was found in broiler breeders, broilers and layers. According to the questionnaire, respiratory symptoms were present in infected broiler breeders (3/7 farms; 42.8%), infected broilers (5/7 farms; 71.4%) and infected layers (1/5 farms; 20%). Mean mortality for infected broiler breeders, broiler and layer farms, was 5.4%, 2.8% and 9.8%, respectively. One out of six infected broiler breeder, and 2/7 infected broiler farms currently used antibiotics (tylosine, Pharmasin®, Eurovet and doxycycline, Soludox®, Eurovet). Nevertheless, we were able to detect viable *C. psittaci*. A high stocking density (number of chickens/m^2) was significantly related to the risk of acquiring chlamydiosis ($p = 0.006$). The negative farm was the only with no poultry farms nearby (<4 km). Plus, it was the only farm with a very long sanitary period (8 weeks), which is the period in-between emptying the barn, cleaning, disinfection and restocking (usually 1 to 2 weeks). However, the latter two observations were not significantly related to the risk of chlamydiosis in chickens ($p = 0.08$ and 0.157, respectively). Antibiotics were not used at the time of sampling.

Table II-2: Pharyngeal excretion of viable *C. psittaci* by poultry (n = 10 per farm) and poultry workers (n = 1 or 2 per farm).

Farm Type	Poultry					Poultry workers		
	Farms positive/total	Culture score*		Positive % within flock	Genotype*	Culture score*		Genotype*
		Mean ± SD	Range			Mean ± SD	Range	
Broiler	7/7	1.7 ± 0.6	0–5	94	D (7/7)	1.9 ± 1.4	1–5	D (7/7)
Layer	5/5	1.8 ± 0.5	0–5	94	A (1/5); D (4/5)	2.1 ± 0.9	1–3	A (1/5); D (4/5)
Broiler Breeder	6/7	1.8 ± 0.2	1–4	100	D (6/6)	1.8 ± 0.7	1–3	D (5/6); C, D (1/6)

*Within culture-positive farms.

Table II-3: Viable *C. psittaci* and perceived health status in poultry farms

C. psittaci in broiler farms (n=10 per farm)				Health status of broilers (questionnaire)			
Age (weeks)	Positive (%)	Score (mean ± SD)	Genotype	Density (no. m^{-2})	Mortality (%)	Respiratory symptoms (% broods)	Antibiotic use (% broods)
2	100	2.8±0.8	D	19.0	2.0	10	10 (doxy*)
<1	100	2.0±1.2	D	18.0	3.5	25	0
1	60	0.9±1.0	D	14.0	3.5	15	0
2–3	100	1.2±0.6	D	20.0	2.8	10	10 (tylo†)
2–3	100	2.0±1.3	D	10.0‡	3.0	10	0
2–3	100	1.3±0.5	D	20.0	2.0	0	0
5	100	1.7±0.9	D	19.5	3.0	0	0
C. psittaci in layer farms (n=10 per farm)				Health status of layers (questionnaire)			
32	100	1.8 ± 1.0	A	7.0	5.0	0	0
37	100	1.5 ± 0.8	D	5.0‡	NA	0	0
39	100	2.4 ± 1.0	D	9.0‡	7.0–30.0	0	0
41	100	2.1 ± 1.3	D	9.0‡	10.0	10	0
74	70	1.1 ± 1.4	D	9.0‡	4.0	0	0
C. psittaci in broiler breeder farms (n=10 per farm)				Health status of broiler breeders (questionnaire)			
2	100	1.4 ± 0.8	D	10.0	2.0	100	0
31	0	0.0 ± 0.0		7.0	NA	0	0
34	100	2.1 ± 1.2	D	16.5	5.0–10.0	0	0
42	100	2.0 ± 0.9	D	7.2	10.0	10	0
48	100	1.8 ± 1.0	D	6.5	9.3	0	0
50	100	1.6 ± 0.5	D	NA	1.5	0	0
50	100	1.9 ± 1.0	D	9.0	1.2	10	10 (doxy*)

NA, Not available.
*Doxycycline.
†Tylosin.
‡Chickens have access to outside area.

Table II-4: *C. psittaci* perceived health status and psittacosis-compatible symptoms in farm employees

	Viable *C. psittaci*		Personnel data			Current health status		Yearly medical complaints†					Confirmed pneumonia (no. of years ago)
	Score	Genotype	Period	Time (h day^{-1} except for *)	Aves at home	Current symptoms	Antibiotic treatment	Fl	Re	GI	Ey	De	
Broiler farm employees													
Male	5	D	27 years	2*	–	–	–	F^1‡, M^1	NPC^1	S^1, D^1	–	–	–
	1	D	20 years	7	Layers	–	–	–	–	–	–	–	–
	1	D	2 years	7	Birds	–	–	F^1, M^2	–	–	–	–	–
	1	D	15 years	2	Layers	–	–	F^1, M^1	NPC^2	–	–	–	–
	1	D	12 years	1	–	–	–	M^2	–	–	–	R^2	3
	2	D	20 years	1	–	–	–	F^1, M^3	–	V^1	E^1	–	–
	1	D	30 years	2	–	–	–	–	PC^3	–	–	–	19
Female	4	D	25 years	8	–	–	–	F^3, M^3	PC^1	B^1, D^3	–	R^1	–
	3	D	13 years	3	–	–	–	F^2, M^2	NPC^2	–	–	–	2 (pleuritis)
	1	D	30 years	7	–	Cold	–	Every production round a cold at ± 5 weeks					–
Broiler breeder employees													
Male	2	D	15 years	2	–	–	–	–	NPC^2	–	–	–	–
	1	D	7 years	1	–	–	–	F^1, M^1	PC^1	V^1, S^1, D^1	–	–	–
	2	D	19 years	3	–	–	–	F^1, M^1	NPC^1	S^1	–	–	–
	1	D	4.5 years	8	–	Cold	–	F^1	NPC^2, B^2	–	E^2	–	–
	1	D	27 years	4	–	–	–	–	–	–	–	–	22
	2	D	25 years	8	–	–	–	F^2, M^2	PC^2, B^2	V^2, S^2, D^2	E^2	–	–
	0	–	2 years	1	–	–	–	–	–	–	–	–	–
	0	–	17 years	3	–	–	–	–	–	–	–	–	–
Female	3	D	15 years	2	–	‘Allergic feeling’	–	T^3	NPC^2	–	–	R^1	–
	2	D	7 years	2	–	–	–	F^1, M^2	PC^2	V^1, S^1, D^1	–	R^1	–
	2	D	19 years	4	–	Cold	Augmentin (4 weeks ago)	–	NPC^1	S^1	–	–	–
	1	D	27 years	4	–	–		–	–	–	–	–	–
	3	D + C	30 years	8	–	–		F^2, M^2	PC^2	V^2, S^2, D^1	–	–	–

Table II-4: Cont

Layer farm employees													
	Viable *C. psittaci*		Personnel data			Current health status		Yearly medical complaints†					Confirmed pneumonia (no. of years ago)
	Score	Genotype	Period	Time (h day^{-1} except for *)	Aves at home	Current symptoms	Antibiotic treatment	Fl	Re	GI	Ey	De	
Male	3	D	40 years	1	–	–	–	F[1], M[2]	NPC[1], DB[1]	–	–	–	
	3	D	7 years	5	–	–	–	M[2]	NPC[1]	–	–	–	
	1	D	12 years	0.5 *	–	–	–	–	NPC[3], Ex[3]	–	–	–	
	2	D	2 months	0.5 *	Ducks, geese	Cold	–	F[1]	–	–	–	–	
	3	A	17 years	3	–	–	–	F[2]	–	S[2], D[2]	–	R[2]	
Female	3	D	24 years	3	–	–	–	F[1], M[3]	NPC[1], B[1]	–	–	–	
	1	D	23 years	4	–	–	–	–	–	–	–	–	
	1	NA	3 years	3	–	–	–	F[2], M[2]	–	–	E[2]	–	–

NA, not applicable.
*Hours week^{-1}.
†Fl, Flu-like; F, fever; M, myalgia; T, tired-fatigue; Re, respiratory; NPC or PC, (non-) productive cough; B, painful breathing; Ex, morning expectoration; GI, gastrointestinal; V, vomiting; D, diarrhoea; S, stomach ache; Ey, eye; E, painful eyes; De, dermatologic; R, non-specific rash.
‡1, Once or twice; 2, repeatedly; 3, frequently.

3.2 Zoonotic transmissions

The study population consisted of 11 women and 20 men and the average age was 42 years. Three of 31 farmers (9.6%) were vaccinated against human influenza. None were infected by ACC. However, 29/31 (93.5%) of humans were *C. psittaci*-positive by both culture and the *C. psittaci*-specific nested PCR. *C. psittaci* genotype D (n=26), genotype A (n=1) and a mixed genotype D plus C infection (n=1), was discovered in farmers. Genotyping revealed no result for one sample. This sample originated from a female employee of a layer farm which only kept chickens (Table II-4). Thus, *C. psittaci* zoonotic transmission was detected on all but one examined chicken farm.

Many *C. psittaci* positives were found, but only four of them (13.7%), who were non-smokers and had no allergies, currently experienced respiratory diseases (coughing, n = 3 and/or rhinitis, n = 1; sinusitis, n = 1; severe bronchitis, n = 1). They were all infected with genotype D, and the person with bronchitis was currently treated with Augmentin® (Glaxo Smith Kline). We informed the farmers and their physicians of the diagnostic results.

Humans (n=2) working in the *C. psittaci*-negative farm never had respiratory complaints, while 25/29 positive farmers (86.2%), reported yearly medical complaints potentially related to psittacosis (Table II-4). Four out of 31 farmers (12.5 %) mentioned that they had pneumonia after starting to keep chickens (Table II-4).

No potential risk factor like age, gender, living in the direct environment of the farm, number of years employed in the sector, daily time in contact with chickens, pet animals, smoking behavior and medical complaints was significantly related to psittacosis.

4. Discussion

We examined the occurrence of *C. psittaci* on 19 Belgian chicken farms, as well as zoonotic transmissions of these pathogens to farmers because *C. psittaci* is now (re)-emerging in chickens. Limited reports from 1960 to 2000 suggest that chickens are less sensitive to *C. psittaci* infections. However, during the last decade, *C. psittaci* has been detected and isolated from chickens raised in Australia, Belgium, China, France and Germany (Yang *et al.,* 2007; Gaede *et al.,* 2008; Zhang *et al.,* 2008; Laroucau *et al.,* 2009; Robertson *et al.,* 2010; Zhou *et al.,* 2010; Dickx *et al.,* 2011). Recently, Yin *et al.,* (2012), proved Hill-Evans postulates for *C. psittaci* genotype B and D strains isolated from Belgian and French broilers. Less is known about *C. psittaci* genotypes infecting chickens. Up

to now, genotypes B, C, D, F and E/B have been found in chickens (Gaede *et al.,* 2008; Zhang *et al.,* 2008; Dickx *et al.,* 2010; Zhou *et al.,* 2010; Yin *et al.,* 2012).

C. psittaci is apparently not the only emerging chlamydial pathogen in chickens. Laroucau et al., (2009), discovered a new chlamydial agent in chickens raised in France, designated atypical chicken *Chlamydiaceae* (ACC). Remarkably, ACC-positive chickens appeared healthy, but the discovery of ACC coincided with three cases of atypical pneumonia in French poultry workers (Laroucau *et al.,* 2009), warranting the need for epidemiological surveillance in chickens. Since then, ACC has been found in chickens raised in China, Croatia, Germany, Greece and Slovenia (Zocevic *et al.,* 2012). This is why we also included the recently developed ACC-specific real-time PCR in our epidemiological study.

C. psittaci was highly prevalent in chickens and humans. *OmpA* genotyping revealed the presence of genotypes A, C and especially D. To our knowledge, this is the first time that genotype A and only the third time that genotype D, has been identified in chickens. Genotype A is most often found in *Psittaciformes* (cockatoos, parrots, parakeets, lories) and is frequently being transmitted from pet birds to humans. Genotype A has also been isolated from turkeys and wild birds (Van Loock et al., 2005; Verminnen et al., 2006; Geigenfeind et al., 2011; Kalmar et al., 2013). Thus, the pathogen is not restricted to *Psittaciformes* and was probably never noticed before in chickens. However, genotype B and D seem to be most prevalent in chickens. Genotype D is most often found in turkeys, but recently has been associated with zoonotic transfer from chickens to slaughterhouse employees (Dickx *et al.,* 2010). Genotype C has primarily been isolated from ducks and geese, but has been found once before in chickens, namely in China (Zhang et al., 2008). Atypical chicken *Chlamydiaceae* were not detected in chickens, suggesting that ACC are currently not widespread in Belgian chicken flocks, at least when compared to *C. psittaci*. However, we cannot exclude the absence of this emerging chlamydial agent in our chicken flocks. Respiratory disease was present, albeit not on all, *C. psittaci*-infected farms. Respiratory disease was most frequently present on broiler farms, followed by broiler breeder and layer farms. Only broiler and broiler breeder farms claimed to use antibiotics (tylosine, Pharmasin®, Eurovet and doxycycline, Soludox®, Eurovet). Antibiotic usage in European poultry has decreased over the last few years (Moulin *et al.,* 2008; BelVet-SAC report 2012; http://www.belvetsac.ugent.be/), but antibiotics are still frequently used without proper diagnosis; among these are the ones active against *C. psittaci*, with the risk of creating tetracycline resistance as occurred for *Chlamydia suis* (Dugan *et al.,* 2004).

Interestingly, a high stocking density (number of chickens/m^2) was the only risk factor that was positively correlated with the occurrence of *C. psittaci* in chickens. This finding was no surprise, as *C. psittaci* transmission most often occurs from one bird to another bird close by.

As for chickens, ACC were not detected in farmers. However, viable *C. psittaci* were present in 93.5% of the farmers. Genotypes A, C and, as in chickens, especially genotype D were discovered in the farmers. In our study, genotype C (most frequently found in *Anseriformes*; ducks and geese) was not detected in chickens, but we cannot exclude the presence of genotype C on the farm, as only 10 chickens were sampled. Zoonotic transmissions of genotypes A, C and D, and even mixed genotype A, C and D infections in poultry workers, have been observed before by Dickx and Vanrompay (2011), examining employees of a turkey and chicken hatchery. Thus, *C. psittaci*-infected chickens present a substantial zoonotic risk. One human sample could not be genotyped, which could indicate the presence of a new genotype. Attempts to grow the strain to a higher bacterial titre for *ompA* sequencing failed.

Humans (n= 2) of the *C. psittaci*- negative farm never had respiratory complaints, while 25/29 (86.2%) of humans, all working on *C. psittaci*-positive farms, reported yearly medical complaints potentially related to psittacosis (Table II-4). Four out of 31 farmers (12.5 %) mentioned in the questionnaire that they had pneumonia after starting to keep chickens, which was higher than the yearly rate of 8 in 1000 pneumonia cases in Belgium. It is likely that chicken farmers are regularly infected, creating immunity, which protects them against severe disease. However, yearly complaints about fever and respiratory disease were of interest (Table II-4). Whether farmers become carriers, clinical consequences and the importance of co-infections with other human respiratory pathogens are unknown.

Preventing avian chlamydiosis in poultry is difficult because of the endemic nature of the bacteria, the long-term survival of the bacteria in organic material, the intermittent shedding and the many asymptomatic carriers (Pelle-Duporte & Gendre, 2001). An all-in, all-out rearing regime, with thorough cleaning and disinfecting between broods is obligatory. *C. psittaci* is highly susceptible to heat and disinfectants (quaternary ammonium compounds, house-hold bleach) but is resistant to drying, acids and alkali (Smith *et al.,* 2005). Access of wild birds to the animals or food should be prevented. Equipment should be regularly cleaned and disinfected when used for several barns at the farm.

Personal protective measures involve a good hand hygiene protocol and protective clothing, including gloves and an air filter full-face mask. A transition room should be available where protective clothing may be kept. The two most important collective protective measures are ventilation and cleaning. Natural or mechanical ventilation should try to prevent aerosol accumulation and cross-contamination between the different barns. Even continuous disinfection (although expensive) of the air in the barns could be considered. Education and training are very important to guarantee that the preventive measures are well understood and performed (Deschuyffeleer *et al.,* 2012).

5. Conclusion

Despite the governments' obligation to assess any biohazard in the workplace, knowledge on *C. psittaci* and especially ACC in chickens is still relatively undeveloped and a specific risk assessment in poultry production remains to be established. Many health care providers are not familiar with psittacosis, especially with its occupational and zoonotic character. An occupational physician assigned to modern, vertically integrated poultry farming, covering the complete poultry production ranging from the feeding mill to processing facilities, could conduct a campaign to raise general awareness and to inform poultry workers on collective and personal protective measures. The occupational physician should address local physicians with a written document as this may lead to an early diagnosis and treatment in poultry workers (Deschuyffeleer *et al.,* 2012). However, most benefit is to be expected from an efficient avian *Chlamydia* vaccine.

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Chapter III

Innate immune response in chicken HD11 cells elicited by Chlamydia psittaci

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This chapter is accepted as:

Lagae S., and Vanrompay D. Innate immune response in avian macrophages elicited *by Chlamydia psittaci*. Vlaams Diergeneeskundig tijdschrift 84: 133-141.

Abstract

Chlamydia psittaci is a gram-negative obligate intracellular bacterium which mainly infects birds and mammals. Not much is known on innate immunity initiated by *C. psittaci*. This study is on chicken HD11 cell activation and expression of cytokine, chemokine, caspase-1, iNOS and TLR genes during the early phase and mid-cycle period of the developmental cycle of the highly virulent *C. psittaci* strain 92/1293. *C. psittaci* significantly augmented transcript levels for all genes investigated, especially during mid-cycle. These results demonstrate a robust innate immune response of chicken macrophage-like cells by a *C. psittaci* infection.

1. Introduction

In birds, *C. psittaci* replicates in epithelial cells and macrophages of the avian respiratory tract, which can result in a systemic infection (Vanrompay et al., 1995). They possess a unique biphasic developmental cycle, thereby switching between a metabolically inactive infectious state, the elementary body (EB) and a metabolically active non-infectious state, the reticulate body (RB). Following attachment of EBs to the host cell membrane and the subsequent internalization, EBs start to differentiate into RBs within an inclusion which is derived from the host cell membrane during the internalization (Vanrompay et al., 1995). Reticulate bodies start to migrate to the periphery of the inclusion whereby the replication starts by binary fission. Afterwards, RBs can re-differentiate into new infectious EBs followed by the release of those EBs by host cell lysis or reverse endocytosis. In some cases, the developmental cycle can be altered in favour of persistence. Persistent *Chlamydiaceae* or so-called aberrant bodies fail to complete their development from RBs into infectious EBs, but retain their metabolic activity.

Not much is known how the innate immune system of the host is influenced by a *C. psittaci* infection. *C. psittaci* replicates in epithelial cells and macrophages of the avian respiratory tract. Subsequently, *C. psittaci* can be demonstrated in plasma and blood monocytes, resulting in a systemic infection (Vanrompay et al., 1995). Monocytes/macrophages are part of the innate immune system and capable of engulfing and killing pathogens. But probably their most important function is to recruit other myeloid cells, in particular polymorphonuclear phagocytes, to the site of infection by the release of chemotactic cytokines. Macrophages can also activate the adaptive immune response by presenting antigens to $CD4^+$ T cells via class II MHC antigen (Beuttler, 2004). Although monocytes/macrophages play an important role in clearing pathogens, *C. psittaci* as well as other *Chlamydiaceae* are able to survive and even replicate within those cells. Moreover, *C. psittaci* uses blood monocytes as vehicles to establish a systemic infection in birds. As not much is known how the host innate immune is influenced by *C. psittaci*, Beeckman et al., (2010) demonstrated an increased expression of IL-1β and IL-6, CXCLi2, CXCLi1 and CCLi2 following inoculation with the highly virulent *C. psitaci* strain 92/1293 (*ompA* genotype D) at 4h post-infection. Interestingly, exceptionally high IL-10 and no TGF-β4 responses were observed at 4h post inoculation. This could induce macrophage deactivation and NF-κB suppression (Beeckman et al., 2010) and thereby, could dampen innate immunity and promote *C. psittaci* survival in macrophages.

Toll-like receptor (TLR) -mediated recognition of components derived from a wide range of pathogens and their role in the subsequent initiation of innate immune responses is widely accepted (Kawai and Akira, 2011).

The goal of the present study was to examine the expression of cytokines (IL-1β, IL-6, MIF, LITAF (Lipopolysaccharide-induced TNF factor) , IL-12p35, IL-10), caspase-1, GM-CSF, iNOS, chemokines (CXCLi1, CXCLi2, CCLi3, IL-16) and TLRs (TLR2, TLR3, TLR4, TLR5, TLR7, TLR21) during an infection of HD11 cells with the virulent *C. psittaci* strain 92/1293 at different time points.

2. Material and Methods

2.1. Chlamydia and cell lines

The well-characterized virulent *Chlamydia psittaci* strain 92/1293 used in this study was isolated from the lung, spleen and cloaca of a diseased turkey (Vanrompay et al., 1993). The bacterium was grown in Buffalo Green Monkey (BGM) cells as described previously (Vanrompay et al., 1993) and the median tissue culture infective dose ($TCID_{50}$) was determined using the method of Spearman and Kaerber (Mayr et al., 1974).

The HD11 cell line was derived from chicken myelomonocytic cells transformed by the v-myc encoding retrovirus MC29 (Beug et al., 1979). The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 1% L-glutamine, 0.5% gentamicin, 5% Fetal Calf Serum (FCS) and 1% sodium pyruvate (Invitrogen, Merelbeke, Belgium) and were incubated in a humidified atmosphere at 37°C and 5% CO_2.

2.2. Chlamydia psittaci infection of HD11 cells

HD11 cells were seeded in a 25 cm^2 tissue culture flask at a concentration of 300 000 cells/ml and grown for 24h at 37°C and 5% CO_2. The medium was aspirated and 1,5 x 10^6 HD11 cells were infected with *C. psittaci* at a multiplicity of infection (MOI) of 1. Irreversible attachment and cell entry was accomplished by incubating the HD11 cells for 3 hours on a rocking platform at 37°C. The unbound organisms were washed away with DMEM (37°C) and culture medium enriched with 5.5 mg/l glucose (Sigma) was added to each tissue culture flask. Infected cells were incubated until RNA extraction at 2h, 4h, 8h, 12h and 18h p.i.

2.3. Transcription analysis of cytokine, chemokine, caspase-1, iNOS and TLR genes

We determined the innate immune response following *C. psittaci* infection of HD11 cells by examining gene transcript levels of IL-1β, IL-6, MIF, LITAF, IL-12p35, IL-10, caspase-1, GM-CSF, iNOS, CXCLi1, CXCLi2, CCLi3, IL-16, TLR2, TLR3, TLR4, TLR5, TLR7, TLR21 in infected and control HD11 cultures. Specific primers (Table 1) were designed using primer 3 (http://frodo.wi.mit.edu/primer3/) and DINA melt (http://www.bioinfo.rpi.edu/ applications/hybrid) software programs. Specificity of all RT-PCR primers were initially checked by conventional PCR followed by cloning (pGEM-T Easy Vector System, Promega, Leiden, The Netherlands) and DNA sequencing of the inserts (LGC Genomics, Berlin, Germany). As we were not able to design primers, which were 100% specific for IL10, IL12p35 and GM-CSF, probes, kindly provided by P. Kaiser and L. Rothwell (Institute for Animal Health, Compton, Berkshire, UK) were needed to verify the specificity of the amplified targets (Table III-1).

Target	Accession No.	Primer and probe sequence (5'-3')	T_a
HD11 28S rRNA	X59733	F: TTTGGGTTTTAAGCAGGAGGT R: TTGCGACAACACATCATCAGT	58°C
C. psittaci 16S rRNA	CPU68447	F: GTCAAGTCAGCATGGCCCTT R: CCCAGTCATCAGCCTCACCT	58°C
MS2 spike	Unpublished	F: Unpublished R: Unpublished	60°C
IL-1β	Y15006.1	F: CACAGAGATGGCGTTCGTT R: GTGACGGGCTCAAAAACCT	58°C
IL-6	NM_204628	F: AGAAATGCCTGACGAAGCTCT R: CACGGTCTTCTCCATAAACGA	58°C
Caspase-1	AF031351	F: TGCCATGAAGACAAAACTTCC R: TCTACACATCTCCAGCCATCC	58°C
MIF	M95776	F: CAGAACAAGACCTACACCAAGC R: CTAACAAGGAGCCATCCATC	58°C
IL-10	AJ621254	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG P: CGACGATTCGGCGCTGTCACC	55°C
LITAF	AY765397	F: TCCTCACCCCTACCCTGTC R: TCAGAGCATCAACGCAAAAG	58°C
IL-12p35	AJ262751	F: TGGCCGCTGCAAACG R: ACCTCTTCAAGGGTGCACTCA P: CCAGCGTCCTCTGCTTCTGCACCTT	55°C
GM-CSF	AJ621253	F: CCTGGAAGAAATAACGAGTCACTTG R: ACAGGTTTATCCCTGATGTCCAT P: AGCGGCCACAGCAGGTCTGTCC	55°C
CXCLi1	Y14971	F: CCACTGCTTACTGGCTTATCG R: CTTGGGATGGATGAACTTGG	55°C
CXCLi2	NM_205498	F: CTCGCTCTTCTCATCGCATC R: GGCAGCAGTGTCCCATCC	58°C
CCLi3	Y18692	F: AGCCTGCCATCATCTTCATC R: AAACAGCACCTGCCATGAG	58°C
IL-16	NM_204352	F: CTCAGCCCAAAACCATCAGT R: GGTGGCAGTAAGTGGAAAGC	58°C

TLR2	AB046533	F: CCTGGTGTTCCTGTTCATCC R: AGCGTCTTGTGGCTCTTCTC	58°C
TLR3	NM_001011691	F: GGCTAAACGACACTCAAGCA R: GGCGTCATAATCAAACACTCC	58°C
TLR4	NM_001030693	F: TGGCACCTACCCTGTCTTTC R: GGCTTGGAGTGGCTTGTATG	58°C
TLR5	NM_001024586	F: AACTCCCTTCCTTCCCACAT R: AACCTCTCTCCCACACAAGC	58°C
TLR7	NM_001011688	F: ATCAGCACAGGGATGGAAAG R: GGGGAACGGTAGTCAGAAGG	58°C
TLR21	NM_001030558	F: AGAAGCAACCACAGGGAGAA R: AAGCACTTTTGGGGTCCTTT	58°C
iNOS	D85422	F: ACCCACCCAACAACTGCTAA R: GCCCTTGTCCATCTCTTGTC	58°C

Table III-1: Real-time quantitative RT-PCR primers and probes. F: forward primer; R: reverse primer; P: 5'-FAM (5-carboxyfluorescein) + 3'-TAMRA (6- carboxytetramethylrhodamine) probe.

Total RNA from 1,5 x 10^6 infected HD11 cells (MOI 1) was prepared using the Total RNA Isolation Reagent (TRIR, ABgene, Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. RNA from uninfected cells served as negative controls. After RNA extraction, samples were treated with RNase-free amplification grade DNase I (Promega) following the manufacturer's instructions and were confirmed to be DNA-free by performing a PCR for the *C. psittaci* 16S rRNA gene. One microgram of total RNA was reverse transcribed (reverse-IT™ 1st Strand Synthesis, Thermo Scientific) into host cell cDNA using the anchored oligo-dT molecule. Each RNA sample was spiked with 5ng coliphage MS2 control RNA (RNA Control Kit, Thermo Scientific). All experiments were performed in duplicate, with replicates performed at different days.

Following cDNA synthesis, cDNA amplification was performed for 6 cytokine genes, 4 chemokine genes, the caspase-1 gene, the GM-CSF gene, the iNOS gene, 6 TLR genes, the HD11 28S rRNA normalization gene and the MS2 spike. cDNA amplification was performed using the Absolute™ QPCR SYBR® Green Mix (Thermo Scientific). The DNA polymerase was initially activated for 15 min at 94°C. Then 40 cycles of amplification were carried out using the Rotor Gene RG-3000 cycler (Westburg) according to the following cycle profile: DNA was denatured at 95°C for 10 min and during 40 cycles of 95°C for 30s, primers (Table III-1) annealed at 55-60°C for 30s and extended at 72°C for 30s. Program settings included acquisition on the FAM/Sybr channel in the extension step and a gain of six. Quantification was done as described by Beeckman et al. (2008), using standard graphs of the cycle treshold (Ct) values obtained by testing 10-fold serial dilutions (10^9 to 10^1 molecules/µl) of the purified PCR products. All samples and standards were tested in duplicate. Ct-values of the samples were automatically converted into initial template quantities (N_0) by use of the RotorGene software 6.0 (Westburg) using imported standard curves from previous runs. Quantification results of the coliphage MS2 RNA obtained were used to correct for inter-sample variability, while quantification results of the

HD11 28S rRNA were used to correct for cell growth during the developmental cycle. No difference in mRNA level is therefore shown as a fold change of 1.

2.4. HD11 activation assay

Activation of HD11 cells was determined by measurement of the accumulation of nitrite (NO_2^-) in the culture medium at 2h, 4h, 8h, 12h and 18h post infection with *C. psittaci*. One hundred microliter of the collected cell free supernatant was added to equal amount of Griess reagent (Sigma) and incubated for 15 minutes at room temperature. The amount of NO_2^- (NO) was determined by measuring the absorbance of the reaction product with a spectrophotometer (Tecan Genios Plus) at a wavelength of 585 nm. A 10-fold $NaNO_2$ dilution series ranging from 320 µM to 0.3125 µM (in triplicate) was created to generate a standard curve. This standard curve was used to determine the amount of NO_2^- (NO) in the samples.

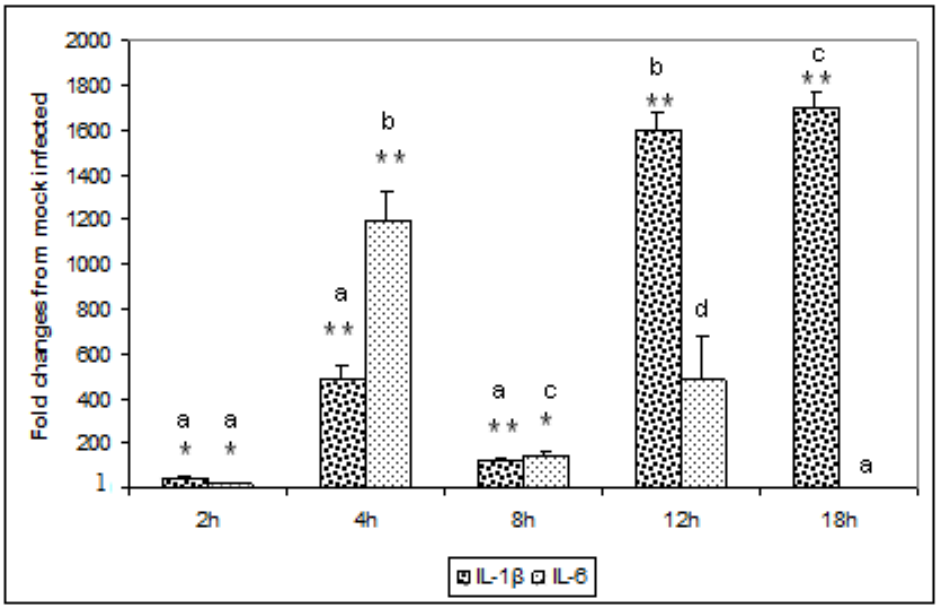
2.5. Statistical analysis

The experiment was performed twice, each time testing all samples in duplicate. Data were pooled for statistical analysis. The mean and standard error mean (SEM) for cytokine-, chemokine-, caspase-1-, GM-CSF-, iNOS-, and TLR-gene transcript levels were calculated. Statistically significant differences ($p<0.05$; $p<0.005$) between the results obtained to investigate the innate immune response elicited by *C. psittaci* was determined using an unpaired Student's *t*-test (SPSS Inc., Chicago Illinois, US). Secondly, an analysis of variance (ANOVA, SPSS Inc.) with post-hoc analysis (both Tukey HSD and Tukey-b) was performed along the time axis to determine significant upregulation time points for cytokine-, chemokine-, caspase-1-, iNOS- and TLR-genes and NO.

3. Results

3.1. Transcription analysis of the caspase-1 gene and cytokines genes

Statistical differences were observed when comparing gene transcript levels of infected cells versus mock infected controls. The influence of an infection on gene expression by comparing gene transcript levels in infected cells versus mock infected controls was examined. The mRNA levels in mock infected controls were presented as an mRNA-fold change of 1. For the pro-inflammatory cytokines IL-1β and IL-6, gene expression upregulation was already noticed at 2h p.i., as compared to the mock infected controls. The upregulation continued, resulting in maximal upregulation of the IL-1β (1671-fold) and IL-6 (1195-fold) gene expression at 18h p.i. and 4h p.i., respectively. IL-1β and IL-6 gene upregulation was most significant from 8h to 12h p.i. The genes for LITAF, IL-12p35 caspase-1, MIF, IL-10 and GM-CSF were all downregulated (mRNA-fold change < 1) during the first 4h p.i, as compared to the results of the mock infected controls. However, at 8h p.i., mRNA-levels for all these genes were comparable to the ones for the mock infected controls, as they were close to a mRNA-fold change of 1. A significant upregulation of the expression of the caspase-1, LITAF, GM-CSF, Caspase-1, MIF and IL-10 genes was observed towards 12h p.i., the beginning of the mid cycle period. Regarding the caspase-1, LITAF , MIF, IL-12p35, IL-10 and GM-CSF genes, upregulation of gene expression was most pronounced for LITAF (23-fold) and IL-12p35 (106-fold). Interestingly, during the mid cycle (from 12h to 18h p.i.), mRNA levels for all genes significantly declined towards the base line level of 1, except for IL-1β, GM-CSF and IL-10. The expression of those genes was significantly upregulated during mid cycle, resulting in a 1671-, 27- and 9.8-fold change in mRNA level, as compared to mock infected controls (Fig. III-1).



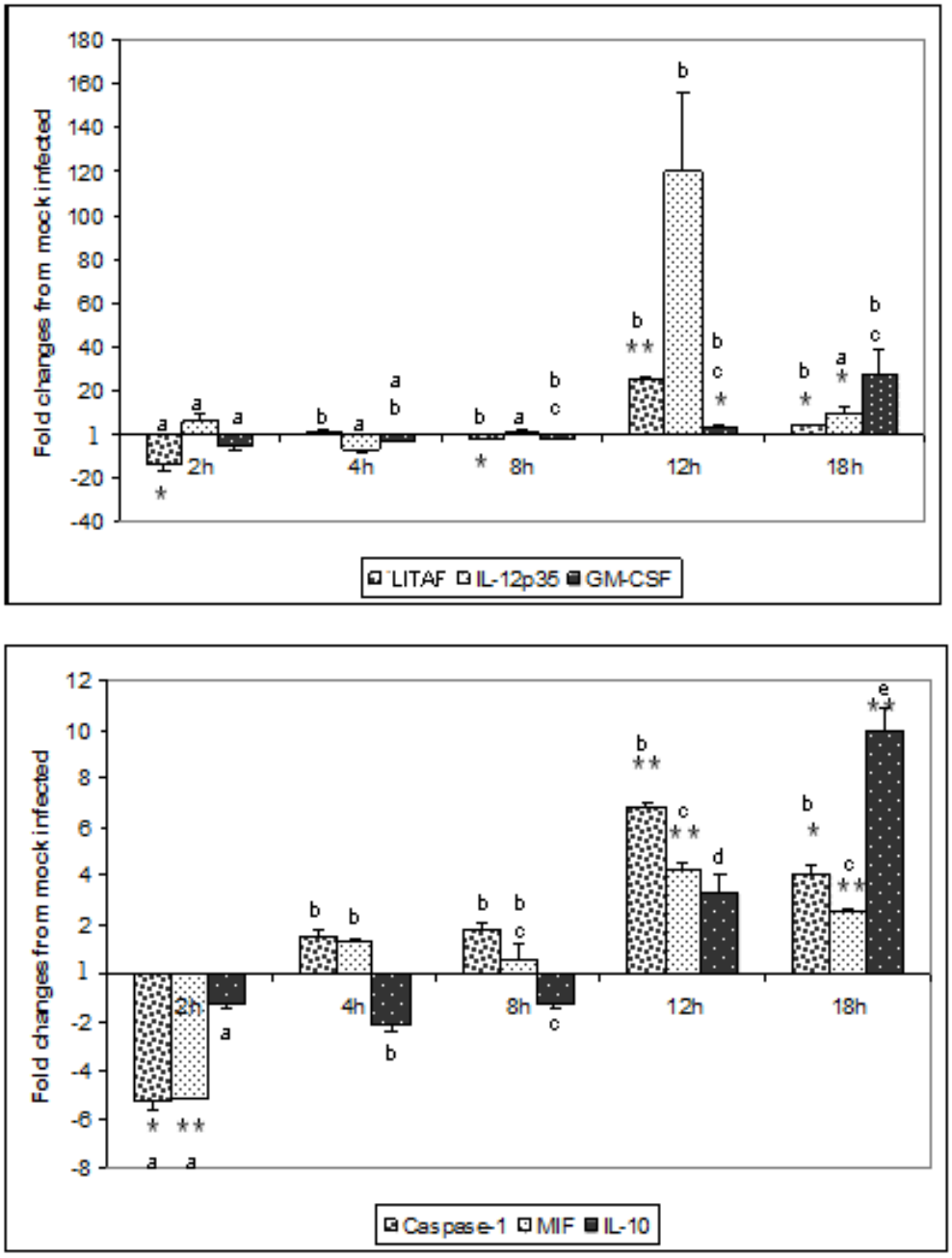


Fig III-1. Cytokine gene expression by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2h, 4h, 8h, 12h and 18h) p.i. Results are presented as fold changes in cytokine mRNA levels compared to mock infected controls. Significant differences between *C. psittaci* infected and mock infected HD11 cells, determined by an unpaired student t test, are indicated by $^{**}P< 0.005$ and $^{*}P<0.05$. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

3.2. Transcription analysis of chemokine genes

We compared the expression of the pro-inflammatory chemokine genes CXCLi1 (K60), CXCLi2 (IL-8), CCLi3 (K203) and IL-16 in *C. psittaci* infected HD11 cells versus mock infected controls. Statistical differences were observed when comparing gene transcript levels for infected cells versus mock infected controls. All chemokine genes, except for the IL-16 gene, were significantly upregulated during the early phase of the bacterial reproduction cycle (85-fold for CXCLi1, 66-fold for CXCLi2 and 89-fold for CCLi3). During mid-cycle, the gene expression upregulation continued (1493-fold for CXCLi1, 471-fold for CXCLi2 and 767-fold for CCLi3). For the IL-16 gene, a significant, but rather moderate expression upregulation was noticed no earlier than mid-cycle (10-fold rise and 2.2-fold rise at 12 and 18h p.i., respectively) (Fig. III-2).

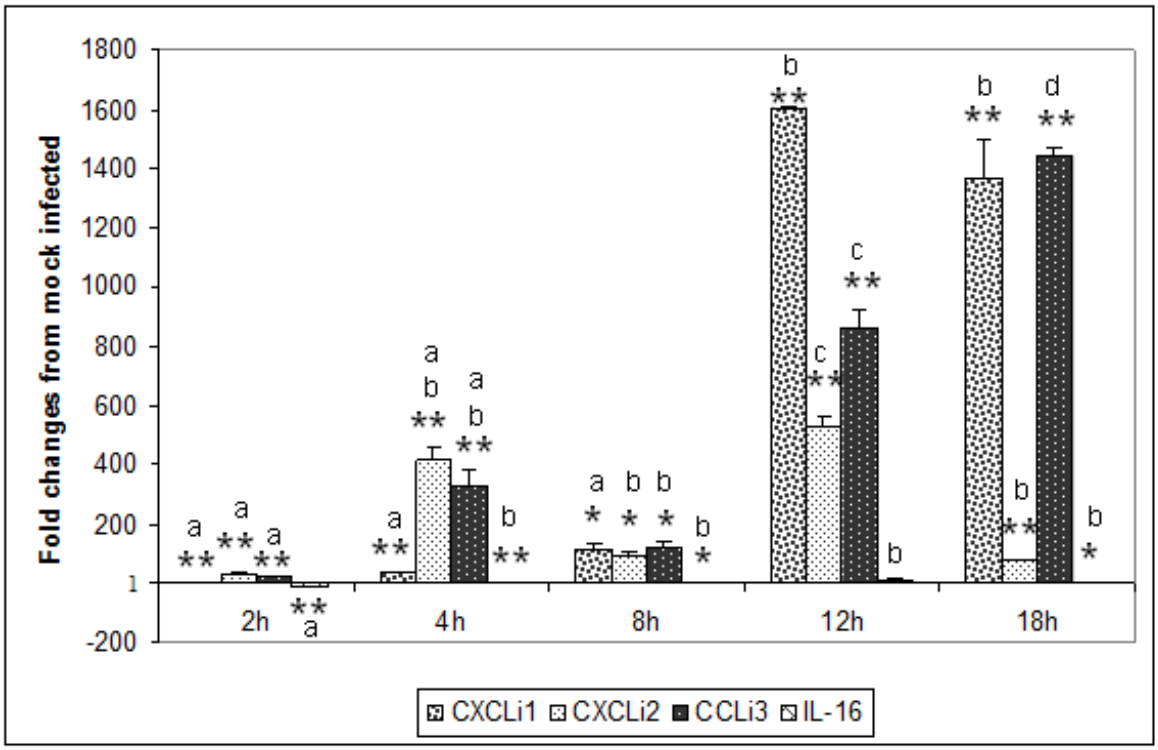
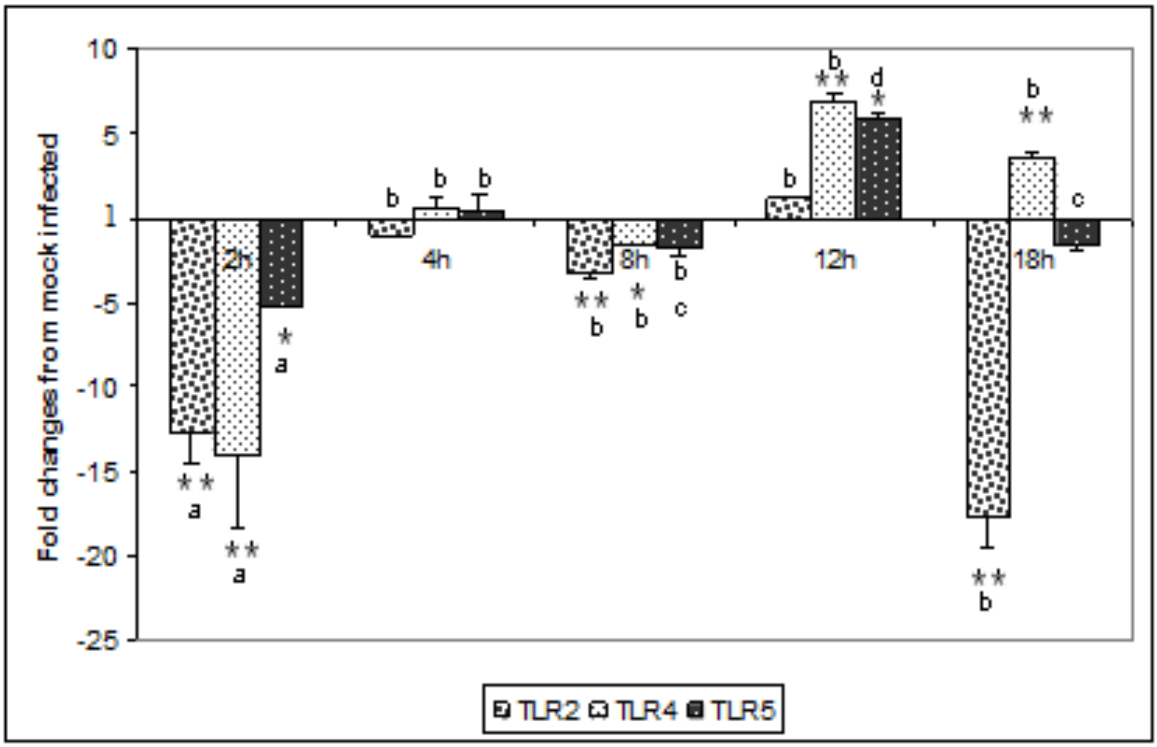


Fig III-2. Chemokine gene expression by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2h, 4h, 8h, 12h and 18h) p.i. Results are presented as fold changes in cytokine mRNA levels compared to mock infected controls. Significant differences between *C. psittaci* infected and mock infected HD11 cells, determined by an unpaired student t test, are indicated by **P< 0.005 and *P<0.05. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

3.3. Transcription analysis of TLR genes

We compared the expression of six known avian TLR genes; TLR2, TLR3, TLR4, TLR5, TLR7 and TLR21 in *C. psittaci* infected HD11 cells versus mock infected controls. During the early phase of the developmental cycle, TLR gene expression was significantly downregulated, as compared to mock infected controls. TLR21 was significantly upregulated (3.6-fold rise) at 8h p.i. In contrast, the expression of all TLR genes, with the exception of the TLR2 gene, was significantly upregulated during mid-cycle (especially, at 12h p.i.). Gene expression upregulation was rather moderate for TLR3 (5.9-fold rise), TLR4 (6.2-fold rise), TLR5 (5.2-fold rise) and TLR7 (4.5-fold rise) genes, whereas gene expression upregulation was more pronounced for the TLR21 gene (34-fold rise) (Fig. III-3).



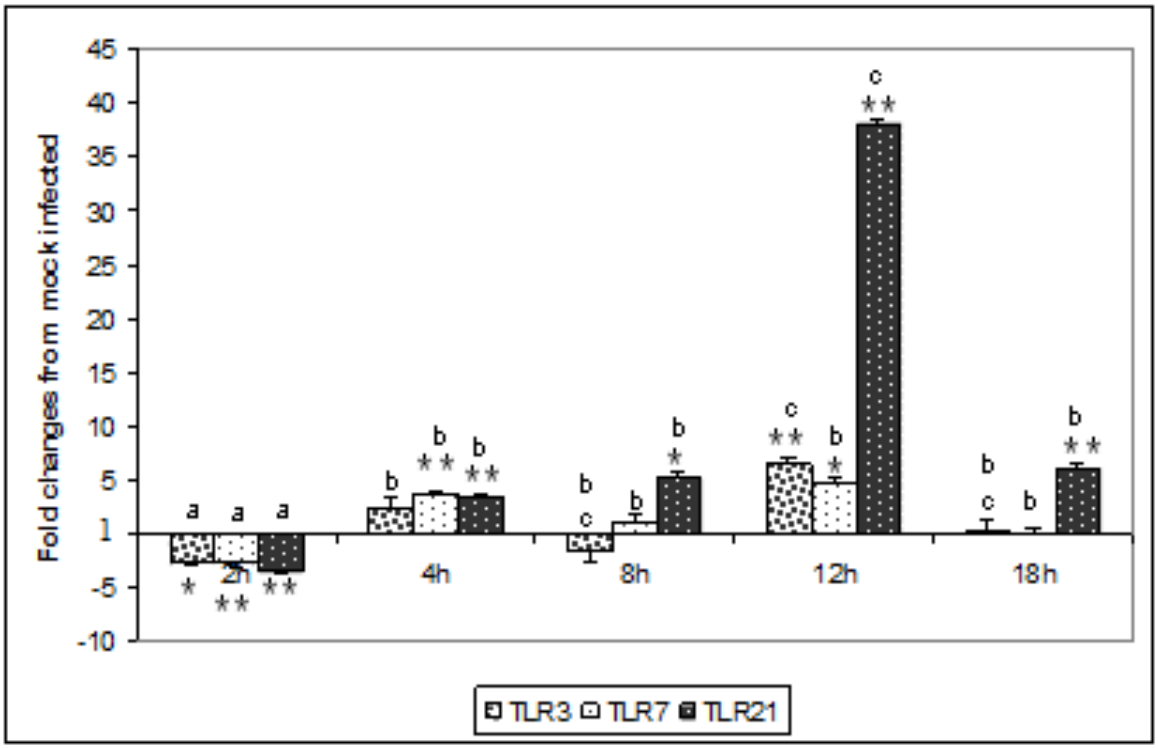


Fig III-3. TLR gene expression by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2h, 4h, 8h, 12h and 18h) p.i. Results are presented as fold changes in cytokine mRNA levels compared to mock infected controls. Significant differences between *C. psittaci* infected and mock infected HD11 cells, determined by an unpaired student t test, are indicated by **P< 0.005 and *P<0.05. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

3.4. Transcription analysis of the iNOS gene and HD11 activation assay

Activation of HD11 cells by *C. psittaci* was evaluated by comparing iNOS gene transcription and NO (NO_2^-) production in infected versus mock infected cells. The expression of the iNOS gene was significantly upregulated during mid-cycle resulting in a 344.6-fold rise of the mRNA level at 18h p.i. The same was observed for the NO production resulting in 91.02 µM ± 4.06 at 18h p.i. versus 1.32 µM ± 0.17 for the mock infected controls (Fig. III-4).

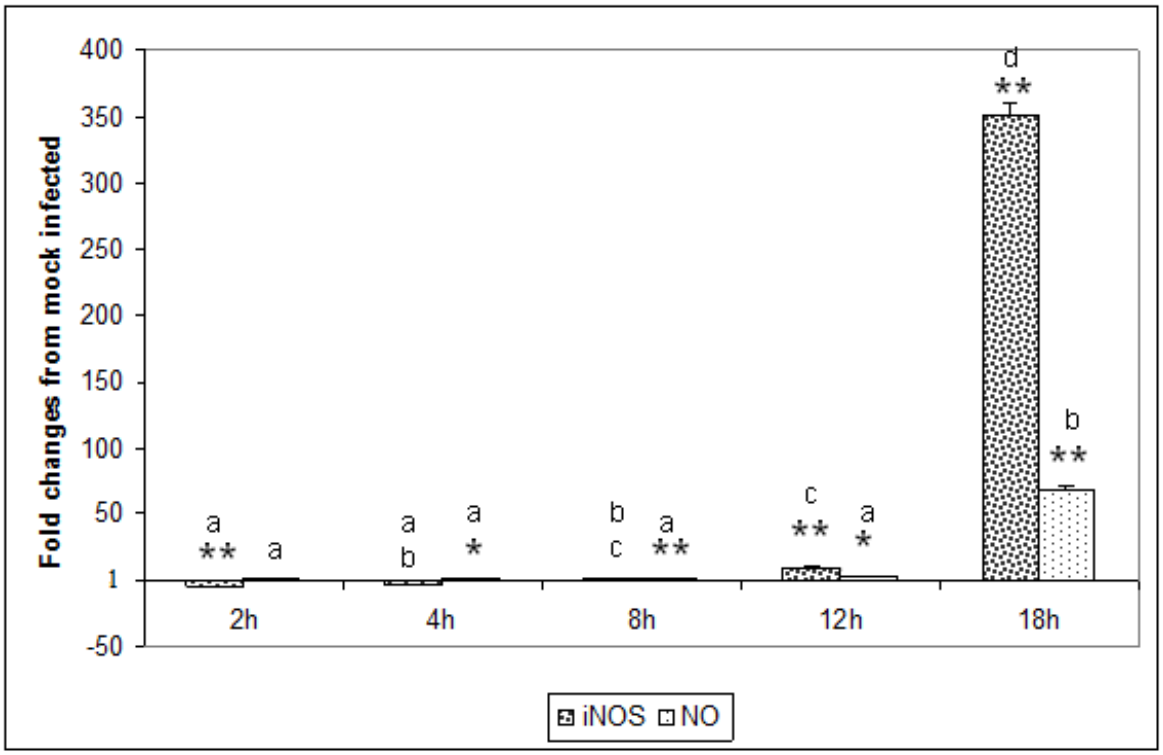


Fig III-4. iNOS gene expression and NO production by HD11 cells infected with *C. psittaci* (MOI=1)) at different time points (2h, 4h, 8h, 12h and 18h) p.i. Results are presented as fold changes in cytokine mRNA levels compared to mock infected controls. Significant differences between *C. psittaci* infected and mock infected HD11 cells, determined by an unpaired student t test, are indicated by **P< 0.005 and *P<0.05. Significant upregulation or downregulation for every cytokine, determined by an ANOVA test, is indicated by a letter. Error bars in all figures represent the standard error mean between two independent experiments performed in duplicate.

4. Discussion

As a member of the obligate intracellular *Chlamydiaceae* family, *Chlamydia psittaci* engages in an intimate relation with respiratory epithelial cells and macrophages. Not much is known of the innate immunity during a primary *Chlamydia* infection. Former studies on chlamydial immunology mainly focused on adaptive immunity against *C. trachomatis*, *C. muridarum*, *C. pneumoniae*, and *C. caviae* whereas it is becoming increasingly clear that the innate immune response influences the migration and activation of immune cells, thereby directing the adapative immune response (Germain, 2004). Very few studies have investigated the innate immune system of the avian respiratory tract (Ariaans et al., 2008; Sarmento et al., 2008 and Wang et al., 2006) and only one study has examined innate immunity to *C. psittaci* in its natural host cell, the respiratory epithelial cell or avian macrophage (Beeckman et al., 2010), although knowledge on the innate immune mechanisms to *C. psittaci* infections and chlamydial antigens is crucial to understand the pathogenesis of, and immunity to this zoonotic pathogen.

The objective of this study was to examine the innate immune response generated after an avian *C. psittaci* infection in a matched avian host cell line. The use of natural host cells in *in vitro* experiments is important, as earlier demonstrated by Roshick et al. (2006).

The current study focused on activation and expression of cytokine and TLR genes by HD11 cells during the early phase (2-8h p.i.) and mid-cycle (12-18h p.i.) period of the developmental cycle of the highly virulent *C. psittaci* strain 92/1293 (*ompA* genotype D). We performed this study on avian macrophage-like cells as it is well known that macrophages play a key role in directing the innate immune response during infection.

First, the expression of inflammatory cytokine genes in *C. psittaci* infected HD11 cells was investigated. Genes encoding the NF-κB-regulated pro-inflammatory cytokines IL-1β and IL-6 were highly expressed during *C. psittaci* infections of avian macrophage-like cells. mRNA levels for both genes showed a significant upregulation at 4h post infection. The same mRNA-fold changes were obtained by Beeckman et al. (2010), using the same model, but inoculating *C. psittaci* at a multiplicity of infection (MOI) of 100 instead of 1 and monitoring cytokine production at 4h p.i. Thus, the expression of the IL-1β and IL-6 genes early on during the *C. psittaci* developmental cycle seemed to be MOI independent. A continuously augmenting IL-1β and IL-6 gene expression upregulation was observed leading to the highest IL-1β and IL-6 gene expression levels during mid- (18h p.i.) and early-cycle (4h p.i.), respectively.Continuously augmenting IL-1β and IL-6 gene expression upregulation was also observed using other *in vitro* models, like for instance *C. muridarum* in primary mouse macrophages (Prantner and Nagarajan, 2009) and *C. trachomatis* in human monocytes/macrophages and THP-1 cells (Bas et al., 2008). In our study, the IL-1β mRNA levels continuously augmented in infected cells, but they were only accompanied by caspase-1 gene upregulation from mid-cycle onwards. IL-1β protein expression is controlled at the posttranslational level, since it requires cleavage of pro-IL-1β by the host protease caspase-1. The effector protein CopB (Fields et al., 2005) could play a role in caspase-1 activation because the homologous T3S translocator proteins in *Shigella* (IpaB) and *Salmonella* (SipB) (Hersh et al., 1999; Hilbi et al., 1998; Thirumalai et al., 1997) have been shown to co-localize with caspase-1 and are necessary and sufficient for its activation. Beeckman et al. (2008), examined the expression of *C. psittaci* T3S effector genes including *copB1* (but not *copB2*). *copB1* was expressed late (>24h p.i.) during the developmental cycle.

Expression of the LITAF-gene, another NF-κB-regulated pro-inflammatory cytokine, was not upregulated till the beginning of the mid-cycle period and this 23-fold upregulation corresponded with a 6.2-fold increase in TLR4 expression. This is in contrast with the study of Prantner and Nagarajan

(2009) in murine macrophages, who observed the highest induction of LITAF mRNA early during infection (8h p.i.) and ascribed this to dominant TLR2-MyD88 signaling.

The importance of the contribution of TLR2 or TLR4 signaling in chlamydial inflammation is still a matter of debate. Joyee and Yang (2008) stated in their review on the role of TLRs in immune responses to chlamydial infections that, although chlamydial LPS and hsp are recognized by TLR4, intact organisms stimulate innate immune cells mainly through TLR2. Interestingly, the TLR2 gene is the only TLR gene in our study, which is not upregulated after internalization of *C. psittaci*, when comparing infected and mock-infected cells. The question rises whether this might present an immune evasion strategy, reducing early secretion of pro-inflammatory cytokines as they may aid in eradicating a chlamydial infection (Darville et al., 2003), or whether this is a crucial mechanism that exists to switch this pathway off to prevent over-amplification of the TLR-2-mediated signal. The gene encoding the chicken macrophage migration inhibitory factor (MIF) was the least upregulated cytokine gene during a *C. psittaci* infection of HD11 cells. This might be beneficial for the pathogenesis of the infection, as high MIF levels could negatively influence the spreading of *C. psittaci* from the lungs to various tissues throughout the body Unlike mammalian MIF, avian MIF alone does not promote the expression of IL-1β, IL-6, IL-12 and IL-8 or NO production in HD11 cells. This only occurs in previously stimulated (primed) cells ([Bernhagen et al., 1994] and [Kim et al., 2010]). Thus, in our study MIF probably plays no role in enhancing cytokine and/or chemokine expression by HD11 cells.

IL-12 gene expression was actually downregulated in the early phase of the infection and it became highly (106.7-fold) upregulated during mid-cycle. A study of Agrawal et al. (2009) showed that IL-12 is involved in protection against *C. trachomatis*.

C. psittaci infection downregulated the expression of the anti-inflammatory IL-10 gene during the early phase of the chlamydial developmental cycle. This is in contrast with our former study, examining IL-10 expression only at 4h p.i. (Beeckman et al., 2010). Previously, we found a 581-fold upregulated IL-10 mRNA level at 4h p.i. This can most likely be attributed to the MOI, which was 100 times higher in the former study.

Similar with the downregulation of the IL-10 gene early in the infection, other genes like IL-12, GM-CSF, LITAF , MIF, caspase-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-7 and TLR21 were also significantly downregulated, suggesting an immune evasion strategy induced by *C. psittaci*.

The pro-inflammatory chemokines CXCLi1, CXCLi2 and CCLi3 were highly expressed, especially during mid-cycle. CXCLi1, CXCLi2 and CCLi3 mediate neutrophil, heterophil and monocyte attraction to the place of infection. Buchholz and Stephens (2008) revealed that the endogenous CXCLi2 response induced by *C. trachomatis* was dependent upon NOD-1 PRR signaling. As TLR-21 gene expression was

significantly upregulated by *C. psittaci*, gene expression analysis for additional intracellular PRR, like NOD-1 will be performed in future experiments. Expression of IL-16 was upregulated by *C. psittaci* during mid-cycle. According to Ghigo et al. (2010) IL-16 promotes replication of *Tropheryma whipplei* by inhibiting phagolysosomal fusion. It is possible that IL-16 plays also a role in the inhibition of the fagolysosomal fusion of *C. psittaci* by activation of the T3SS.

The expression of all examined TLR genes was down-regulated during the first 4h p.i. Gene upregulation was first observed for TLR21 (3.6-fold at 8h p.i.). The avian TLR21 is an intracellular endosomal nucleotide signaling receptor that senses and responds to bacterial genomic DNA (Keestra et al., 2010). Thus, *C. psittaci* is certainly recognized by intracellular signaling receptors. This might influence the expression of pathogen recognition receptors (PRRs) directly or their downstream signaling. Unfortunately, other intracellular receptors like RIG-I like receptors, NOD-like receptors and inflammasomes are not yet investigated.

In conclusion, our results showed a clearer view on how *C. psittaci* is recognized by HD11 cells and its influence on the host innate immune response. High expression of cytokines, chemokines, iNOS, caspase-1 and GM-CSF genes with a peak during mid cycle of the developmental infection were observed. Further research on other pattern recognition receptors and their pathways is necessary to map the innate immune responses elicited by *C. psittaci* in avian macrophages.

Chapter IV

Biology and the elicited innate immune response of C. psittaci in human macrophages

This chapter will be published as:

Lagae S., Soen B., Saad N., and Vanrompay D. Biology and the elicited innate immune response of *C. psittaci* in human macrophages. In Preparation.

Abstract

Chlamydia psittaci is a Gram-negative obligate intracellular bacterium, causing respiratory infections in birds and humans. The bacterium can survive and even replicate within macrophages, which are phagocytes of the innate immune system involved in the phagocytosis, stimulation of host cell defense and presentation of antigens to activate the adaptive immune response. The development cycle, the T3SS and the innate immunity initiated by *C. psittaci* was investigated in THP1 cells. A rapid early entry was observed followed by an increase in the average area per inclusion between 24h and 48h p.i. resulting in the lyse of the host cell at 48h p.i. The study of the T3SS of *C. psittaci* revealed the continuous expression of IncA. Furthermore, the effector proteins SctW and SctC were also detected during the developmental cycle. The staining data indicate that the T3SS is continuously expressed and active during the infection cycle in THP1 cells.

Less is known of the innate immunity elicited by *C. psittaci*. In this way, it was determined which pattern recognition receptors were involved during infection with *C. psittaci* in THP1 cells. A significant upregulation of TLR4, TLR6, TLR3, TLR9+, NLRP3, ASC, RIG-I, NOD-2 was noticed during infection. Moreover, different cytokines and chemokines such as IL-1β, TNF-α, IL-6, IL-8 and GM-CSF were detected in the supernatant of infected THP1 cells.

1. Introduction

Chlamydia psittaci infect a wide range of avian species. In addition, *C. psittaci* has a tremendous zoonotic potential and all genotypes can readily infect humans via inhalations of nasal and ocular secretions and/or faeces of infected animals (Cray, 2011). Symptoms in humans may vary from unapparent to severe and the infection can even cause death when untreated (Rohde et al., 2010). After inhalation, *C. psittaci* binds and enters epithelial cells and macrophages from the respiratory tract where it starts his unique biphasic developmental cycle alternating between the elementary body (EB) and the reticulate body (RB). In order to enter and survive in the host environment, *C. psittaci* translocate effector proteins into the host cell by using a sophisticated molecular syringe called the type III secretion system (T3SS) (Beeckman et al., 2008). The developmental cycle start with the attachment of EB to the host cell membrane, thereby on entry, translocating an actin-recruiting protein called tarp (translocated actin-recruiting phosphoprotein) into the host cell to induce recruitment and polymerization of actin at the attachment site, assisting the internalization of the bacteria (Carabeo et al., 2002; Subtil et al., 2004, Beeckman et al., 2007). Other effector proteins translocated into the host cell are the inclusion membrane proteins or Incs, in which IncA mediates homotypic fusion of inclusion probably through t_SNARE domains (Rockey et al., 2002; Delevoye et al., 2004). Interestingly, the expression of genes encoding components of the T3SS vary in the developmental cycle in agreement with the alternation between EBs and RBs, the replication of RBs, but it is also dependent on the *Chlamydiae* species, the strain and the host cell used (Slepenkin et al., 2003; Beeckman and Vanrompay, 2010).

As monocytes and macrophages are part of the innate immune system, it is unique that *Chlamydiaceae* can enter and even replicate within those cells. However, multiple inclusions and metabolically active aberrant bodies were observed in monocytes infected with *C. pneumonia* (Airenne et al., 1999). So, *Chlamydiae* appear to become spontaneously persistent following infection of monocytes. Furthermore, the number of infectious progeny of EBs from macrophages was much lower when compared to EBs recovered from an epithelial cell line such as HeLa cells. As *Chlamydiae* has a lower ability to replicate and to generate new metabolically active EBs when compared to epithelial cells and the observation of aberrant RBs, lead to the assumption that monocytes/macrophages may rather serve as carriers for the bacteria to infect other organs and establish a systemic infection (Gaydos et al., 1996; Wolf et al., 2005).

Macrophages are wide distributed throughout the body of the host and they will rarely be far away from infectious organisms introduced by any route. Macrophages are mobile scavenger cells capable of engulfing and killing microbes, but probably their most important function is to supervise through

the elaboration of chemotactic cytokines and thereby recruiting other immune cells to place of infection. They can also activate the adaptive immune response through the presentation of antigens to T-cells via MHC molecules (Beuttler, 2004). Macrophages express a range of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG) -I-like and nucleotide-binding oligomerization domain (NOD) -like sensing receptors, on their surface and in vacuolar and cytosolic compartments capable of sensing PAMPs of *Chlamydiaceae* (Athman and Philpott, 2004). Researchers have observed that all those receptors do play a role in sensing *Chlamydiaceae* and are responsible for the evoked immune response mediated by several cytokines and chemokines. Several reports indicate an important role for NOD-like receptors, especially NOD1, leading to a high IL-8 production during chlamydial infection (Opitz et al., 2005; Shimada et al., 2009; Buchholz and Stephens, 2008). Furthermore, the NLRP3/ASC inflammasome is necessary for the induction of IL-1β through the activation of caspase-1 during chlamydial infection in macrophages and epithelial cells (Abdul-Sater et al., 2009; Shimada et al., 2009; He et al., 2010). However, to generate functional IL-1β proteins, pro-IL-1β must be first formed through the activation of TLRs before caspase-1 can proteolytically cleaves this precursor to the pro-inflammatory cytokine IL-1β. Several studies have showed that different TLRs such as TLR2 and TLR4 are activated after infection with *Chlamydiae* (Prebeck et al., 2001; Bulut et al., 2002; Netea et al., 2002; Shimada et al., 2012).

However, how *C. psittaci* is recognized by human macrophages and the innate immune response that is elicited is unknown. The aim of this study it to investigate the mode of action of the T3SS of *C. psittaci* in THP1 cells and the macrophage response to the chlamydial infection. This study focused on the *in vitro* development of *C. psittaci* in THP1 cells and the elicited immune response by 1) the determination of the characteristics of the replication cycle of *C. psittaci* in THP1 cells, 2) clarifying the role of T3SS in the developmental cycle *of C. psittaci* in THP1 cells and 3) examination of the recognition and the evoked immune response of *C. psittaci* by THP1 cells. A better understanding of the pathogenesis of *C. psittaci* and the elicited innate immune response in human macrophages will lead to a better understanding of the disease in humans and consequent innovative anti-microbial therapies.

2. Material and Methods

2.1. Chlamydia and cell lines

The *Chlamydia psittaci* genotype B strain CP3 used in this study was isolated from a pigeon (Page and Bankowski, 1959). The bacteria were grown in Buffalo Green Monkey (BGM) cells using standard techniques. The EBs and RBs were purified by discontinuous gradient ultracentrifugation using four different concentrations of urografin (60 vol%, 54 vol%, 44 vol% and 32 vol%) mixed with Tris-KCl. Ultracentrifugation (Beckman Coulter, SW40 Ti) was performed at 50 000g for 60 minutes at 4°C. After ultracentrifugation, the 32/44 interphase contained the RBs and the 44/54 interphase contained the EBs. The EBs were collected and the tissue culture infective dose ($TCID_{50}$) was determined by the method of Spearman and Kaerber (Mayer et al., 1974).

THP1 cells, a human monocytic cell line derived from an acute monocytic leukemia patient, were cultured in RPMI1640 + glutamax (Gibcon) medium supplemented with 1% streptomycin and 2% vancomycin and were incubated in a humidified atmosphere at 37°C and 5% CO_2.

2.2. Chlamydia psittaci infection in THP1 cells

THP1 cells were seeded in Chlamydia trac bottles (Bibby Sterilin Ltd., Stone, UK) at a concentration of 300 000 cells/ml and grown for 24h at 37°C and 5% CO_2. To differentiate THP1 monocytes into adherent macrophages a concentration of 50 ng/ml phorbol 12-myristate 13-acetate (PMA) was added at the cell culture medium. After 24h, the medium was aspirated and 300 000 THP1 cells were infected with *C. psittaci* genotype B using different multiplicity of infections (MOIs). Irreversible attachment and cell entry was performed by centrifugation at 1300g for 15minutes at 4°C. After centrifugation, the inoculum was removed and cell culture medium enriched with 5.5 mg/l glucose (Sigma) was added to every trac bottle. The trac bottles were incubated at 37°C.

2.3. Replication, inclusion size and titration of the infectious progeny of C. psittaci in THP1 cells

To determine the inclusion forming units per ml (IFU/ml) and the inclusion size (MFA: mean fluorescence area) of *C. psittaci* in human macrophages, the fluorescence-based digital titration technique described by Beeckman et al. (2009) was used. Different MOIs (MOI of 1, 2, 5, 10 and 20) were investigated at different time points (30min, 1h, 24h, 48h and 6 days) post infection. Every condition was 4 times repeated. Inclusion forming units on each glass slide were stained by a direct immunofluorescence staining (IMAGEN™ *Chlamydia*, Oxoid, Drongen, Belgium) (Vanrompay et al., 1995). The coverslides were examined under the microscope (Olympus BX41, magnification 600x) and

fluorescence was visualized with a Mercury-lamp (EXFO X-cite, series 120 Q). Throughout the cover slip, 50 random fluorescent images were recorded (Olympus XC30 camera) with the Cell^F software. The images were digitally analyzed with an in-house developed ImageJ application and processed with programmed Microsoft Excell macros. The Microsoft Excell macros generated the inclusion forming units per ml (IFU/ml) and the mean fluorescent area (MFA) [pixels^2].

Moreover, the infectious progeny of *C. psittaci* on human macrophages was also titrated. THP1 macrophages were infected at a MOI of 1. At 12h, 18h, 24h and 72 post infection, the supernatant was aspirated. A tenfold dilution series of the supernatant was made and inoculated on BGM cells. After 6 days, the BGM were fixed with methanol and stained with IMAGEN Chlamydia (Oxoid). The $TCID_{50}$/ml was determined using the method of Spaerman and Kaerber (Mayer et al., 1974).

2.4. Immunofluorescence assay of the T3SS of C. psittaci

We tested whether *C. psittaci* genotype B is capable to induce actin polymerization at the site of attachment and entry and whether different components of the T3SS are expressed during the developmental cycle. THP1 cells were seeded at a concentration of 300 000 cells/ml in Chlamydia Trac Bottles and subsequently inoculated at an MOI of 1, performing a centrifugation step at 1300g for 15 minutes at 4°C in order to synchronize irreversible attachment and entry. After centrifugation, the inoculum was removed and 1 ml of culture medium with glucose was added and the cells were incubated at 37°C. The cells were fixated using fixative solution (4% paraformaldehyde in 120 mM sucrose solution) followed by permeabilization with saponin at 15 min, 1h, 4h, 8h, 24h and 48h p.i. (Beeckman et al., 2007). Colocalization between *C. psittaci* and actin polymerization was assessed using AlexaFluor 488 coupled phalloidin (Molecular Probes, Invitrogen) and an indirect staining for *C. psittaci* using a primary antibody which is a rabbit polyclonal antibody prepared against purified EBs, followed by an AlexaFluor 546 labeled goat anti-rabbit conjugate (Molecular Probes).

As T3SS plays an important role in different phases of the chlamydial life cycle, we studied different T3S-related proteins such as SctC, SctW and IncA in THP1 cells using the expression of rabbit polyclonal anti-StC, SctW and IncA – antibodies, followed by an AlexaFluor 546 labeled goat anti-rabbit conjugate (Molecular Probes). *C. psittaci* staining was done using a direct immunofluorescence staining (IMAGEN Chalmydia, Oxoid, Drongen, Belgium).

All dilutions and washing steps were performed with 1% BSA (Sigma) in PBS). The nuclei were visualized in all samples with DAPI or 4',6'-diamidino-2-phenylindole. Coverslips were mounted using Mowiol (Calbiochem, VWR, Haasrode, Belgium) with 0.01% p-phenylenediamine (PPD, Sigma, Bornem,

Belgium). The coverslips were examined under the microscope (Olympus BX41, magnification 600x). All images were recorded and merged with the Cell^F software.

2.5. Transcription analysis of different Pattern Recognition Receptors

We determined the expression of different PRRs following *C. psittaci* infection (MOI = 1) of THP1 cells by examining gene transcript levels of TLR1, TLR2, TLR3, TLR4, TLR6, TLR9, NLRP3/ASC, RIG1 and NOD2 in infected and control THP1 cultures during the early- (2h, 4h, 8h), mid- (12h, 18h 24h) and late- (36h, 48h, 72h) phase of the developmental cycle. Specific primers for the different PRRs found in the literature were used (Table IV-1).

Target	Primer sequence 3' – 5'	T_a	Reference
TLR1	F: CAGTGTCTGGTACACGCA TGGT R: TTTCAAAAACCGTGTCTGTTAAGAGA	60°C	Tamaki et al., 2011
TLR2	F: GGCCAGCAAATTACCTGTGTG R: AGGCGGACATCCTGAACCT	60°C	Tamaki et al., 2011
TLR3	F: TCCATAAAAGCCGAAAGGTG R: TTCCAGAGCCGTGCTAAGTT	60°C	Tamaki et al., 2011
TLR4	F: TCCATAAAAGCCGAAAGGTG R: CTGAGCAGGGTCTTCTTCAC	60°C	Tamaki et al., 2011
TLR6	F: GGATAGCCACTGCAACATCA R: TTGGTTTTCACGGGTAGGTC	60°C	Tamaki et al., 2011
TLR9	F: GCTAGACCTGTCCCGCAATA R: ACACTTGGCTGTGGATGTTG	60°C	Tamaki et al., 2011
NLRP3	F: CTTCCTTTCCAGTTTGCTGC R: TCTCGCAGTCCACTTCCTTT	55°C	Abdul-Sater et al., 2009
ASC	F: AGTTTCACACCAGCCTGGAA R: TTTTCAAGCTGGCTTTTCGT	55°C	Abdul-Sater et al., 2009
NOD2	F: CATGTGCTGCTACGTGTTCTC R: CCTGCCACAATTGAAGAGGTG	60°C	Tang et al., 2011
RIG1	F: TGTTCTCAGATGCCTTGGATG R: CACTGCTCACCAGATTGCAT	55°C	Conceicao et al., 2010
β-actin	F: TCACCCACACTGTGCCCATCTACGA R: CAGCGGAACCGCTCATTGCCAATGG	60°C	Tamaki et al., 2011

Table IV-1: Real-time quantitative RT-PCR primers and probes. F: forward primer; R: reverse primer; T_a: annealing temperature.

Total RNA was extracted from 300 000 cells using the Total RNA Isolation Reagent (TRIR, ABgene, Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. After RNA extraction, samples were treated with RNase-free amplification grade DNase I (Promega) following the manufacturer's instructions and were confirmed to be DNA-free by performing a PCR for the β-actin gene. One microgram of total RNA was reverse transcribed (reverse-IT^{TM} 1st Strand Synthesis, Thermo Scientific) into host cell cDNA using the anchored oligo-dT molecule. The experiment was performed three times and qPCR using SYBRGreen technology was performed in duplo on each sample.

Quantification was done as described by Beeckman et al. (2008), using standard graphs of the cycle treshold (Ct) values obtained by testing 10-fold serial dilutions (10^9 to 10^1 molecules/µl) of the purified PCR products. Ct-values of the samples were automatically converted into initial template quantities (N_0) by use of the RotorGene software 6.0 (Westburg) using imported standard curves from previous runs. Quantification results of the THP1 β-actin were used to correct for cell growth. No difference in mRNA level is therefore shown as a fold change of 0.

2.6. Protein analysis of human cytokines

Different cytokines such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IFN-γ, TNF-α and GM-CSF were investigated in the supernatans of *C. psittaci* infected THP1 cells (MOI=1) during the mid- (12h, 18h, 24h) and late- (36h, 48h and 72h) phase of the developmental cycle using a Multi-Analyte ELISArray Kit (SABiosciences) according to the manufacturer's protocol. The experiment was performed three times and standard curves were used to determine the concentration (ng/ml) for every cytokine.

2.7. Statistical analysis

Every experiment was repeated three times, except the digital titration experiment which was repeated four times. The mean and standard error mean was calculated for every sample. Secondly, an analysis of variance (ANOVA, SPSS Inc.) with post-hoc analysis (both Tukey HSD and Tukey-b) and the non-parametric tests such as Mann-Whitney and Kruskal-Wallis with Bonferroni correction was used to determine significant differences between infected an non-infected THP1 cells and between the different time points in the infected group.

3. Results

3.1. Replication, inclusion size and titration of the infectious progeny of C. psittaci in THP1 cells

The number of IFU/ml and the MFA was obtained using the digital titration technique in order to obtain information on the developmental cycle of *C. psittaci* in THP1 cells.

Regarding at the IFU/ml, a significant decline was observed for all MOIs investigated from 30 min until 48h p.i., followed by an increase from 48h until 6d p.i. However, the observed increase at 6d p.i. is not statistically significant different compared with the other time points investigated, except for a MOI of 2 (Fig. IV-1). Interestingly, regarding at the MFA, a strong peak at 48h p.i. was noticed for all MOIs investigated. Moreover, the MFA of the inclusions at 48h showed significant difference compared with the MFA of the other time points investigated Remarkably, how higher the MOI how smaller the MFA of the inclusions (Fig. IV-2).

Furthermore, a titration was performed on the supernatant of infected THP1 cells (MOI=1) at 12h, 18h, 24h, 36h, 48h and 72h p.i. through inoculation of dilution series of this supernatant on BGM cells. The $TCID_{50}$/ml was the highest (14 600/ml) at 48h p.i., indicating that the developmental cycle last 48h in THP1 cells (Fig. IV-3).

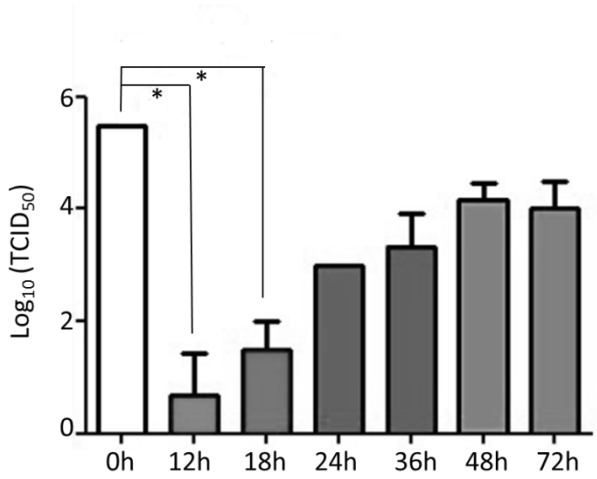


Fig. IV-3: Mean $TCID_{50}$/ml in the supernatant at different time points p.i. (12h, 18h, 24h, 36h, 48h and 72h) of infected THP1 macrophages (MOI=1). Error bars represent the standard error mean between three independent experiments. Statistical significant differences between the different time points were analyzed with the non-parametric Mann-Whitney test.

Fig. IV-1: Number of inclusion forming units (IFU)/ml are shown for given multiplicity of infection (MOI) at different time points p.i. (30 min, 1h, 24h, 48h and 6d). Error bars represent the standard error mean between four independent experiments. Statistical significant differences between the different time points were analyzed with the non-parametric Kruskal-Wallis test with Bonferroni correction.

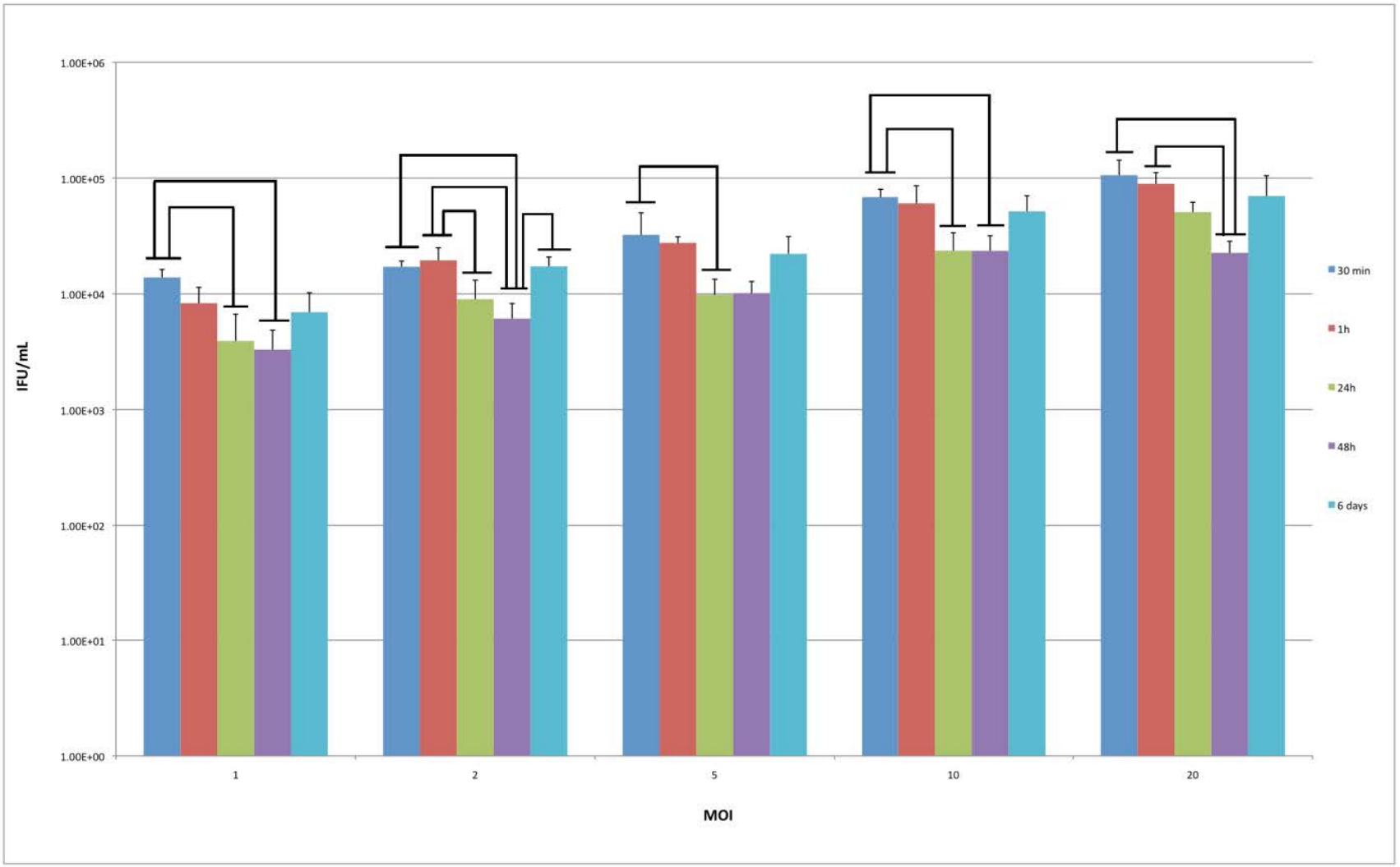
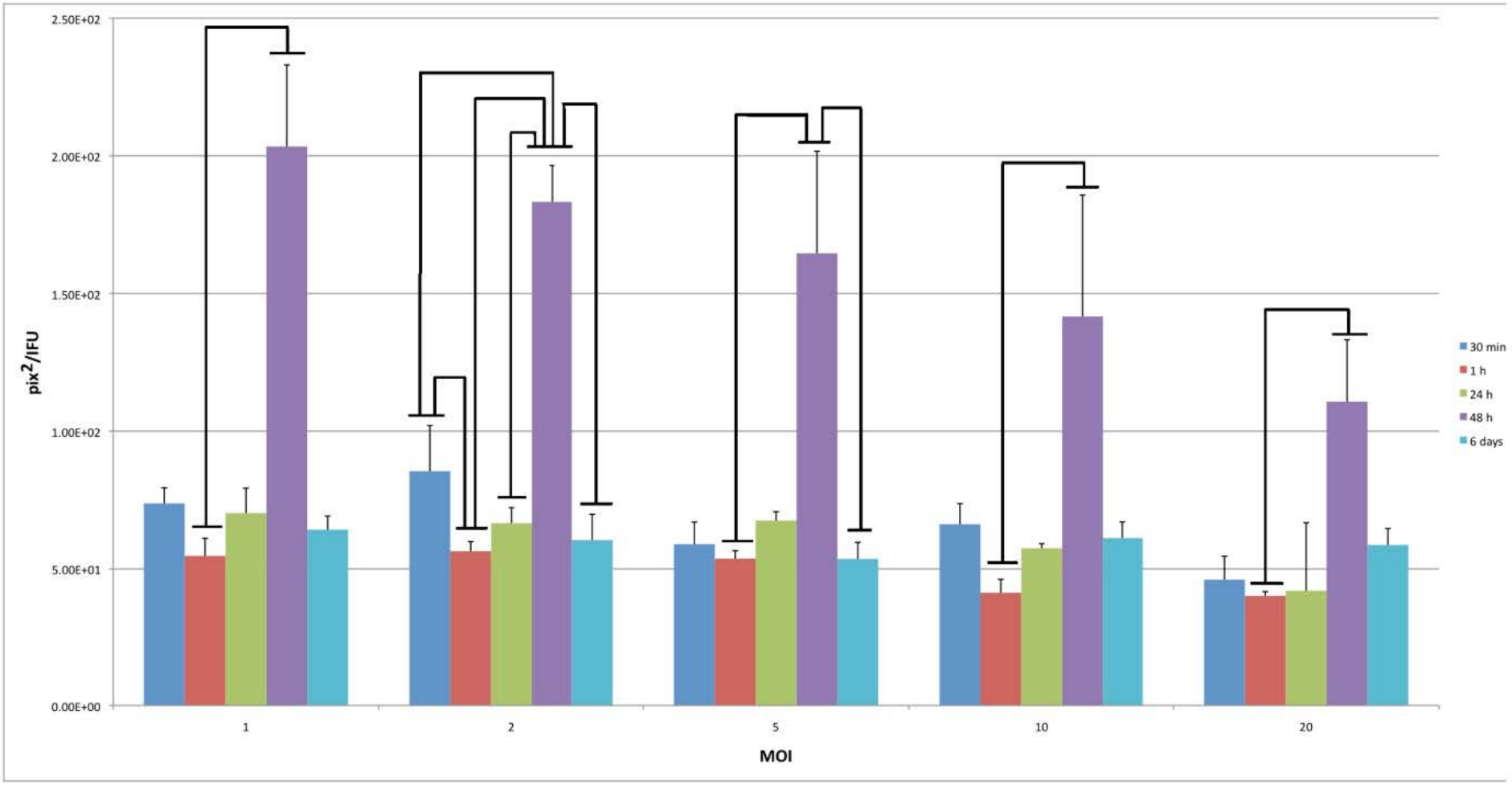


Fig. IV-2: Mean fluorescence area per inclusion (pix²/IFU) are shown for given multiplicity of infection (MOI) at different time points p.i. (30 min, 1h, 24h, 48h and 6d). Error bars represent the standard error mean between four independent experiments. Statistical significant differences between the different time points were analyzed with the non-parametric Kruskal-Wallis test with Bonferroni correction.



3.2. Immunofluorescence assay of the T3SS of C. psittaci

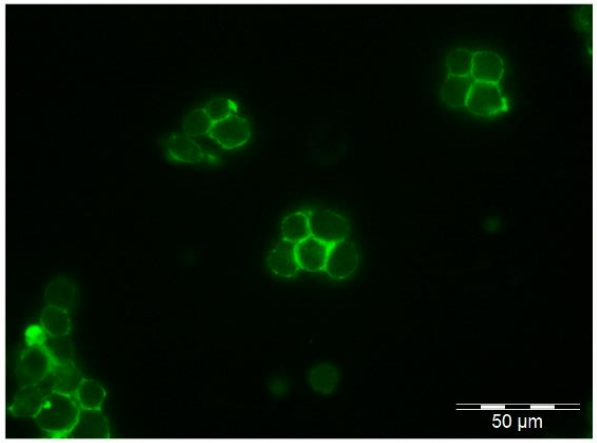
In order to investigate the host cell reaction to infection with *C. psittaci*, THP1 cells were stained for host cell actin and different T3S-related proteins such as SctW, SctC and IncA, bacteria and the nucleus at 15 min, 1h, 4h, 8h, 24h and 48h p.i.

Attachment for *C. psittaci* to the outer cell membrane of THP1 cells was noticed at 15 min p.i. As we used a MOI of 1, only the half of the THP1 cells were infected and the actin polymerization at the site of attachment was not very pronounced. Interestingly, the size and appearance of the cells changes between 1h and 4h p.i., larger and more elongated cells were noticed, which was very apparent at 8h p.i. At 24h p.i., the inclusions were observed at the periphery of the cell, near the cell membrane and at 48h p.i. the majority of the THP1 cells were ruptured, releasing a large numbers of EBs which can again attach and infect macrophages. Interestingly, at this time point it was noticed that the macrophages has a larger and more elongated form. A minority of the cells still has inclusions (Fig. IV-4).

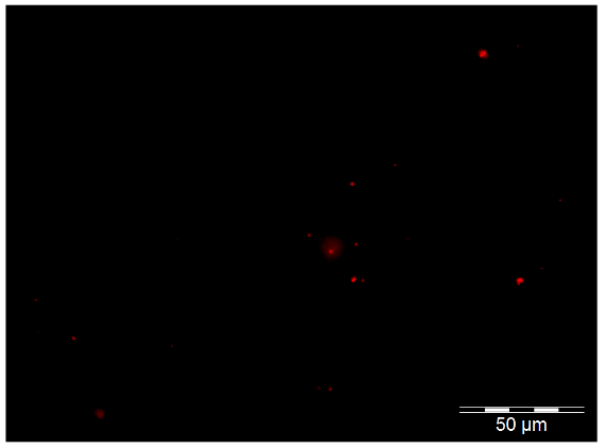
IncA is a T3S effector protein localized into the inclusion membrane. The IncA protein was detected early during the infection (15 min p.i.) in the proximity of the nucleus. However, the majority of the chlamydial organisms did not translocate the effector IncA protein so early in the infection. However, at 1h p.i., almost all chlamydial organisms express the IncA protein in very tight association with the nucleus. Again, at 24h p.i. the inclusion were observed at the cell periphery. Overall, IncA is continuously expressed along with *C. psittaci* throughout the whole developmental cycle (Fig. IV-5).

SctC is essential in the assembly of a functional T3SS as it forms the secreton together with SctW in the bacterial outer membrane. The protein SctC is also very early expressed in the developmental cycle (15 min p.i.). However, we could no longer detect the protein at 1h p.i. From 4h p.i. on the SctC signal reappeared and could be continuously observed until the end of the measurements (Fig. IV-6).

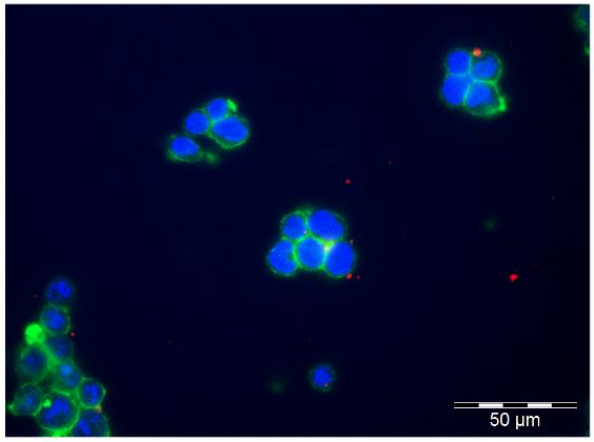
The T3S effector protein SctW regulates the translocation of effector proteins by blocking the needle channel. At 15 min p.i., only one EB was observed showing a SctW signal. In addition, the SctW signal completely disappears from 1h p.i. until 8h p.i. From 24h p.i. on, the signal of SctW re-emerges along with the growth of the inclusion bodies of *C. psittaci* and at 48h p.i only a few inclusions were observed that colocalize with SctW as the number of inclusions was tremendously decreased at that time point (Fig. IV-7).



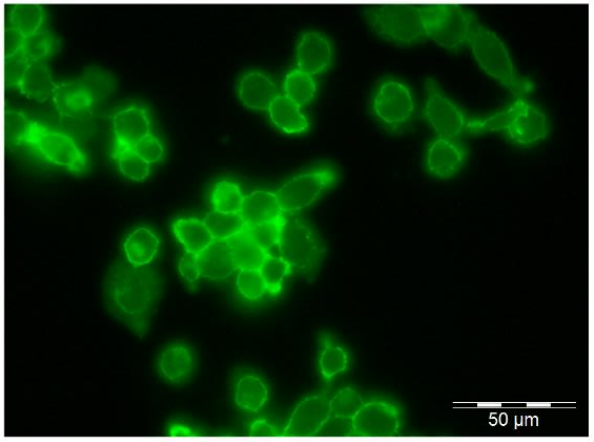
15 minutes p.i. Actin



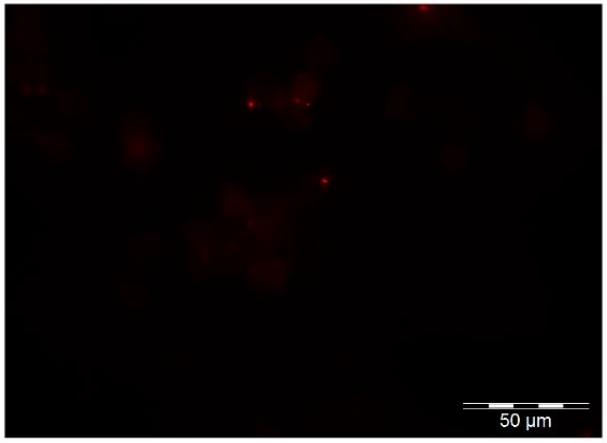
15 minutes p.i. *Chlamydia psittaci*



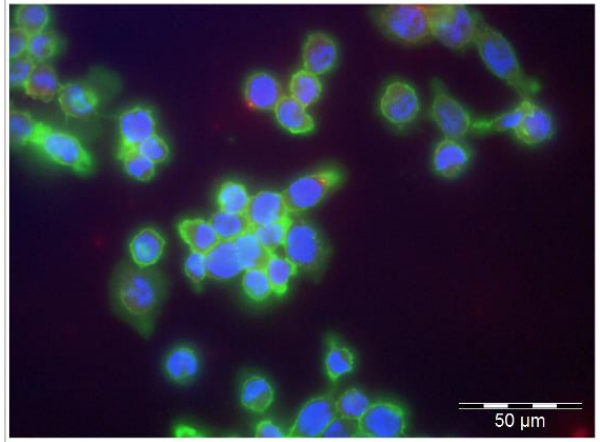
15 minutes p.i. Merged



8 hours p.i. Actin



8 hours p.i. *Chlamydia psittaci*



8 hours p.i. Merged

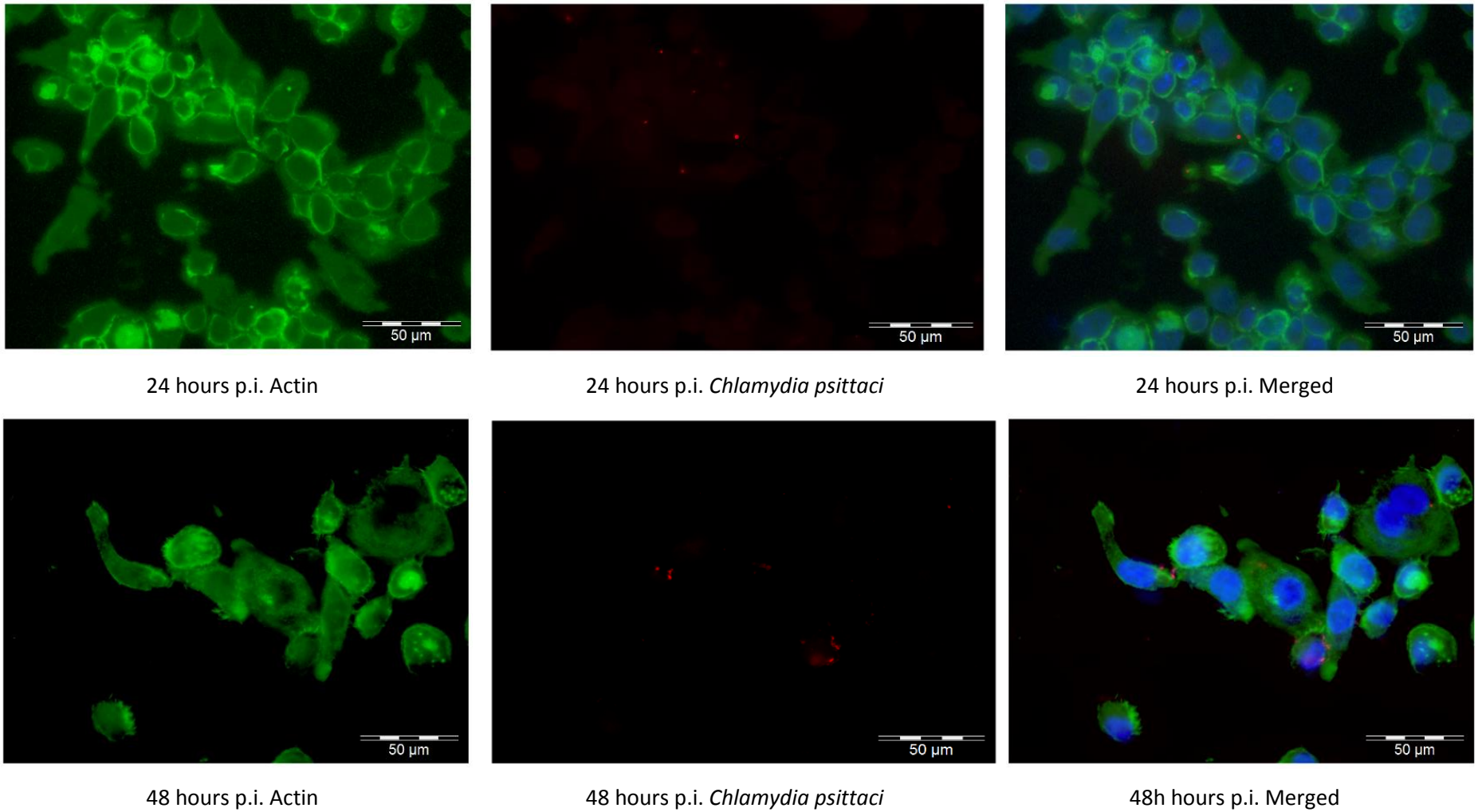
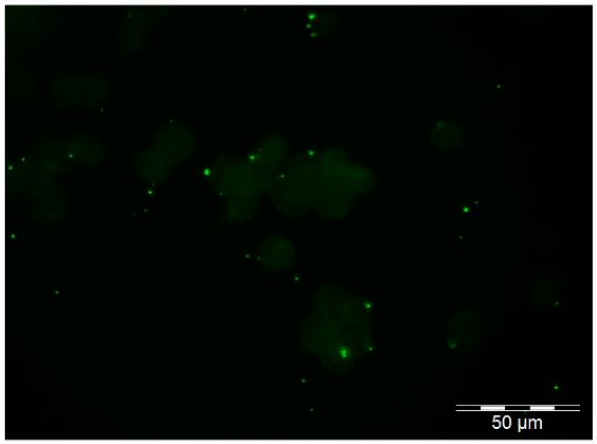
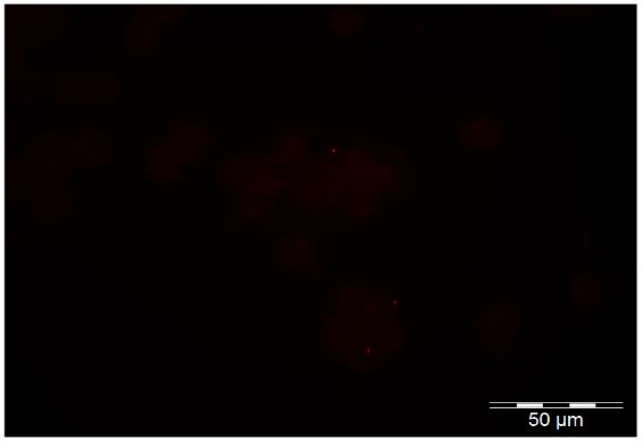


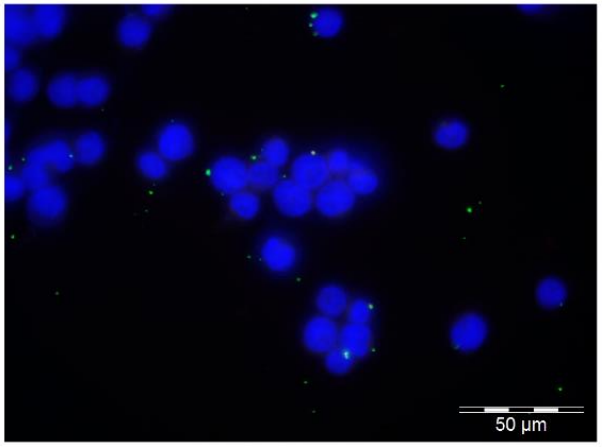
Fig. IV-4: Fluorescent microscopy images of actin staining of THP1 cells infected with *C. psittaci* at MOI 1. Subfigures show C. psittaci (red), actin (green) and merged image with DAPI



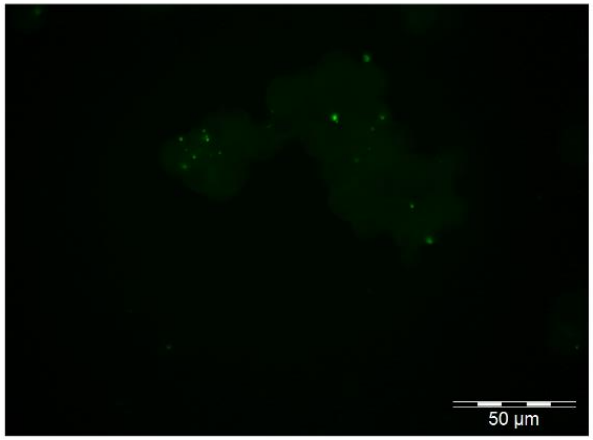
15 minutes p.i. *Chlamydia psittaci*



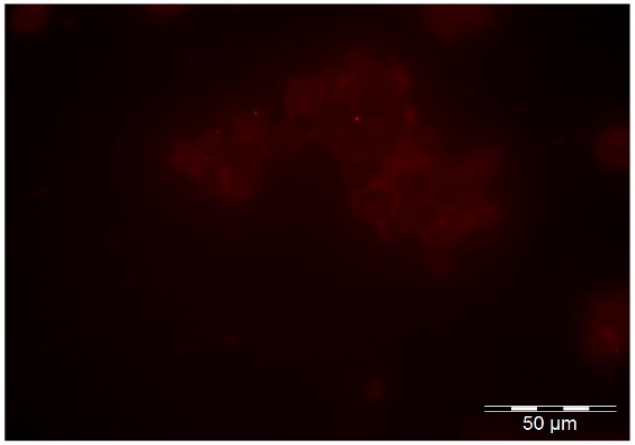
15 minutes p.i. IncA



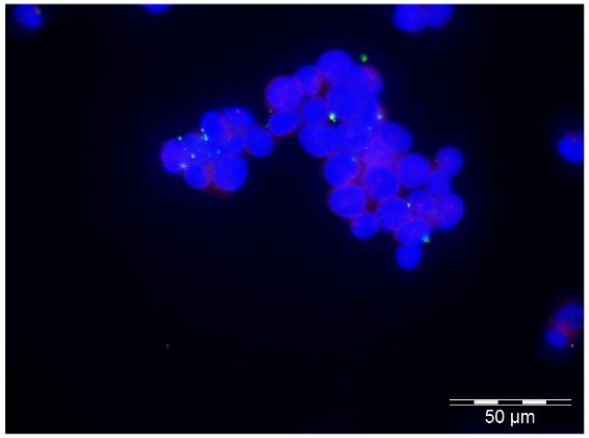
15 minutes p.i. Merged



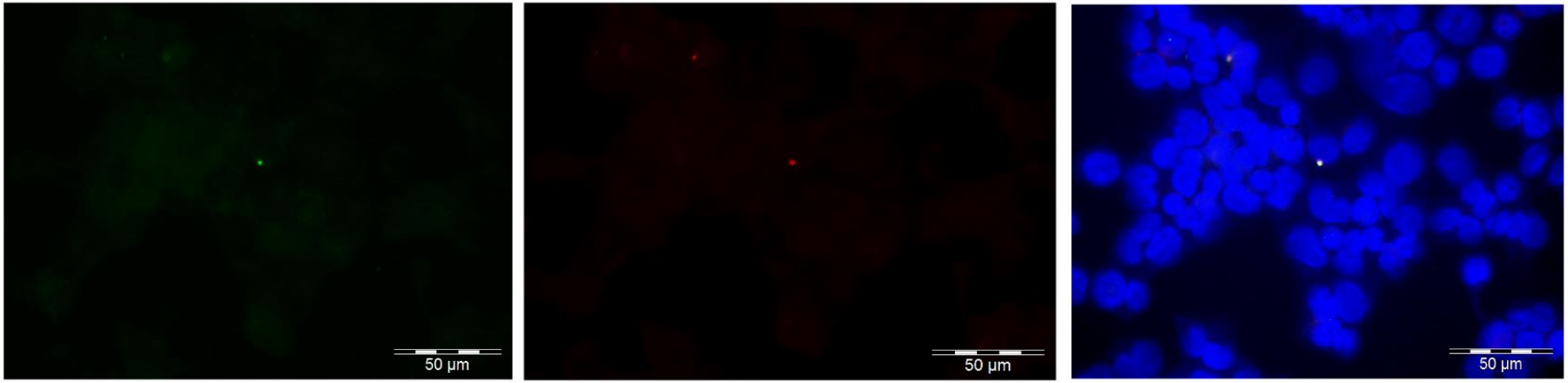
1 hour p.i. *Chlamydia psittaci*



1 hour p.i. IncA



1 hour p.i. Merged



24 hours p.i. *Chlamydia psittaci* 24 hours p.i. IncA 24 hours p.i. Merged

Fig. IV-5: Fluorescent microscopy images of IncA staining of THP1 cells infected with *C. psittaci* at MOI 1. Subfigures show *C. psittaci* (green), IncA (red) and merged image with DAPI

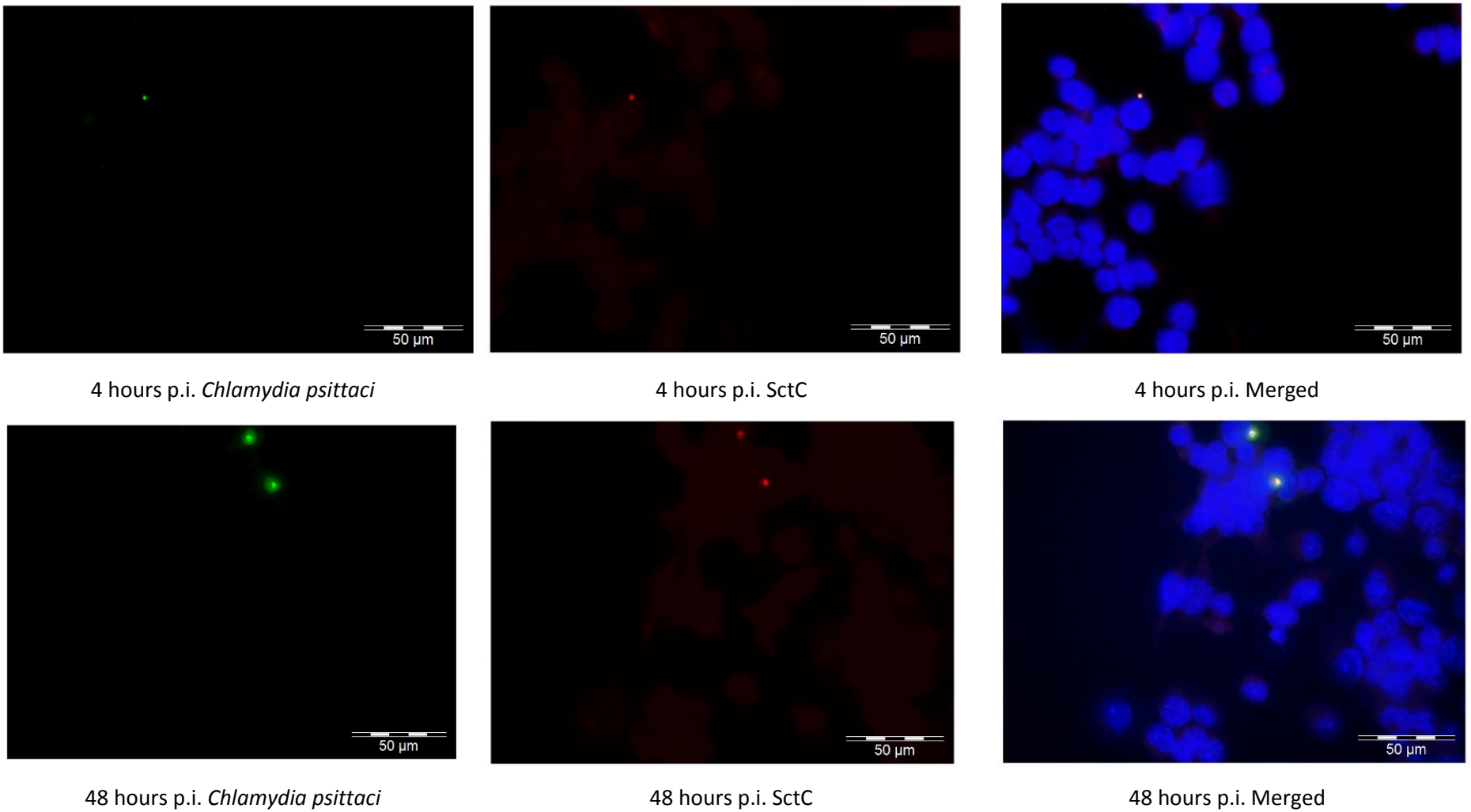
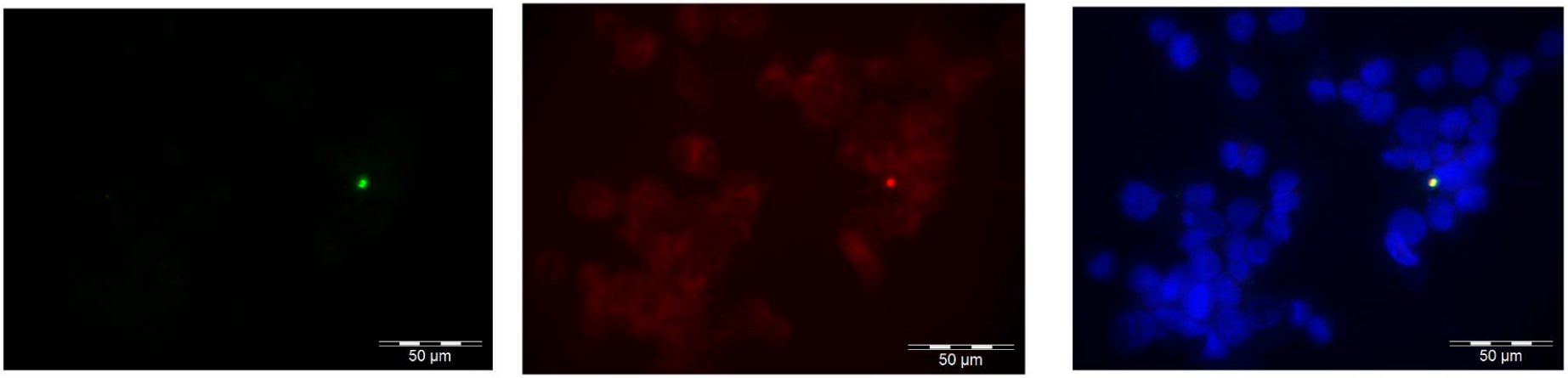


Fig. IV-6: Fluorescent microscopy images of SctC staining of THP1 cells infected with *C. psittaci* at MOI 1. Subfigures show *C. psittaci* (green), SctC (red) and merged image with DAPI.



48 hours p.i. *Chlamydia psittaci* | 48 hours p.i. SctW | 48 hours p.i. Merged

Fig. IV-7: Fluorescent microscopy images of SctW staining of THP1 cells infected with *C. psittaci* at MOI 1. Subfigures show *C. psittaci* (green), SctW (red) and merged image with DAPI.

3.3. Expression analysis of pattern recognition receptors

It was determined which pattern recognition receptors were involved during the infection of *C. psittaci* (MOI =1) in THP1 cells during the early- (2h, 4h, 8h), mid- (12h, 18h, 24h) and late- (36h, 48h, 72h) phase of the developmental cycle. Statistical differences were observed when comparing gene transcript levels of infected THP1 cells versus mock infected THP1 cells. The mRNA levels for mock infected controls were presented as a mRNA fold change of 1.

The PRRs investigated expressed on the surface cell membrane were TLR1, TLR2, TLR6 and TLR4. TLR1 is significantly upregulated at 2h p.i. (118.83 fold) compared to the mock-infected controls. The upregulation continued, resulting in a maximal significant upregulation (237.73-fold) at 4h p.i, followed by a significant decline at 8h p.i. (21.89-fold). Also at later time points such as 18h and 72h p.i. was a significant upregulation noticed in infected THP1 cells compared with the mock-infected controls. Regarding TLR4 and TLR6, a significant upregulation was detected at 2h p.i. (respectively 119-fold and 21.19-fold) followed by a significant downregulation at 8h p.i. (respectively 10.62-fold and 10.84 fold). Furthermore, a significant downregulation was also observed for TLR4 at later time points such as 24h p.i. (2.27-fold) and 72h p.i. (2.03-fold), whereas TLR6 showed a significant upregulation at 18h p.i. (3.92-fold). TLR2 was not expressed during infection with *C. psittaci* in THP1 cells (Fig. IV-8A).

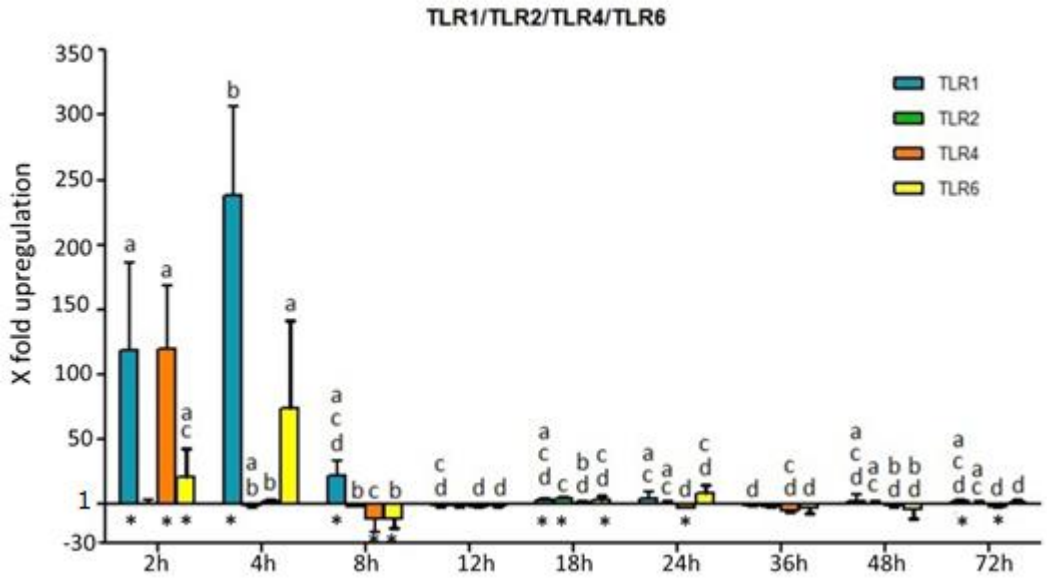
The transcript levels of the intracellular TLR3 and TLR9 were significant upregulated early in the infection cycle at 2h p.i. (3.99-fold and 5.94-fold respectively) and 4h p.i. (6.07-fold and 7.80-fold respectively) followed by a significant downregulation (9.77- fold and 14.13-fold respectively). Interestingly, TLR9 was also upregulated at later time points such as 18h p.i. (11.30-fold) and 24h p.i. (10-87-fold), although there was no significant difference at the latter time point between the infected and the mock-infected cells (Fig IV-8B).

Similar with the intracellular TLR, the transcript levels of the NLRP3/ASC inflammasome was also significant upregulated early in the infection at 2h p.i. (18.54-fold and 17.24-fold respectively) and 4h p.i. (9.30-fold and 16.09-fold respectively). This upregulation was followed by a significant decline. However, at 18h and 72h p.i. the receptor NLRP3 was again significantly upregulated (2.8-fold and 1.72-fold respectively) whereas ASC was significantly upregulated at 24h p.i. (8.58-fold) and 48h p.i. (13-fold) (Fig. IV-8C).

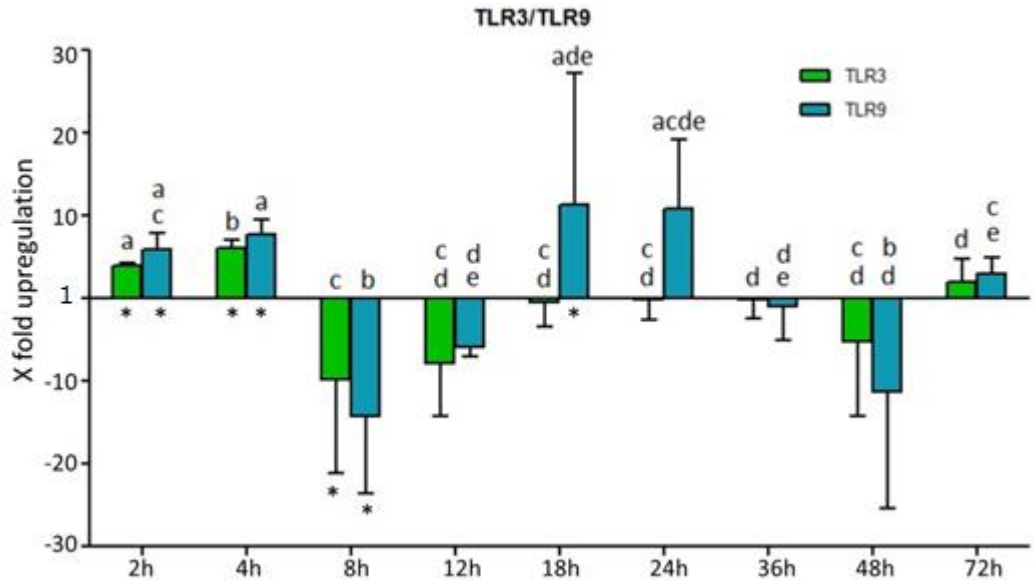
The transcript levels of the intracellular receptors RIG-I and NOD2 were also investigated. NOD2 was together with RIG-I significantly upregulated early in the infection cycle at 2h p.i. (55.80-fold and 18.12-fold respectively) and 4h p.i. (4.09-fold and 20.29-fold respectively) followed by a downregulation for NOD2 at 8h p.i. (6.33-fold) and 12h p.i. (34.81-fold), although the latter time point was not significantly

downregulated in infected THP1 cells compared with mock-infected THP1 cells. Interestingly, a significant upregulation was observed at 18h p.i. (7.95-fold) and 72h p.i. (3.58-fold) for the transcript mRNA levels of RIG-I (Fig. IV-8D).

A



B



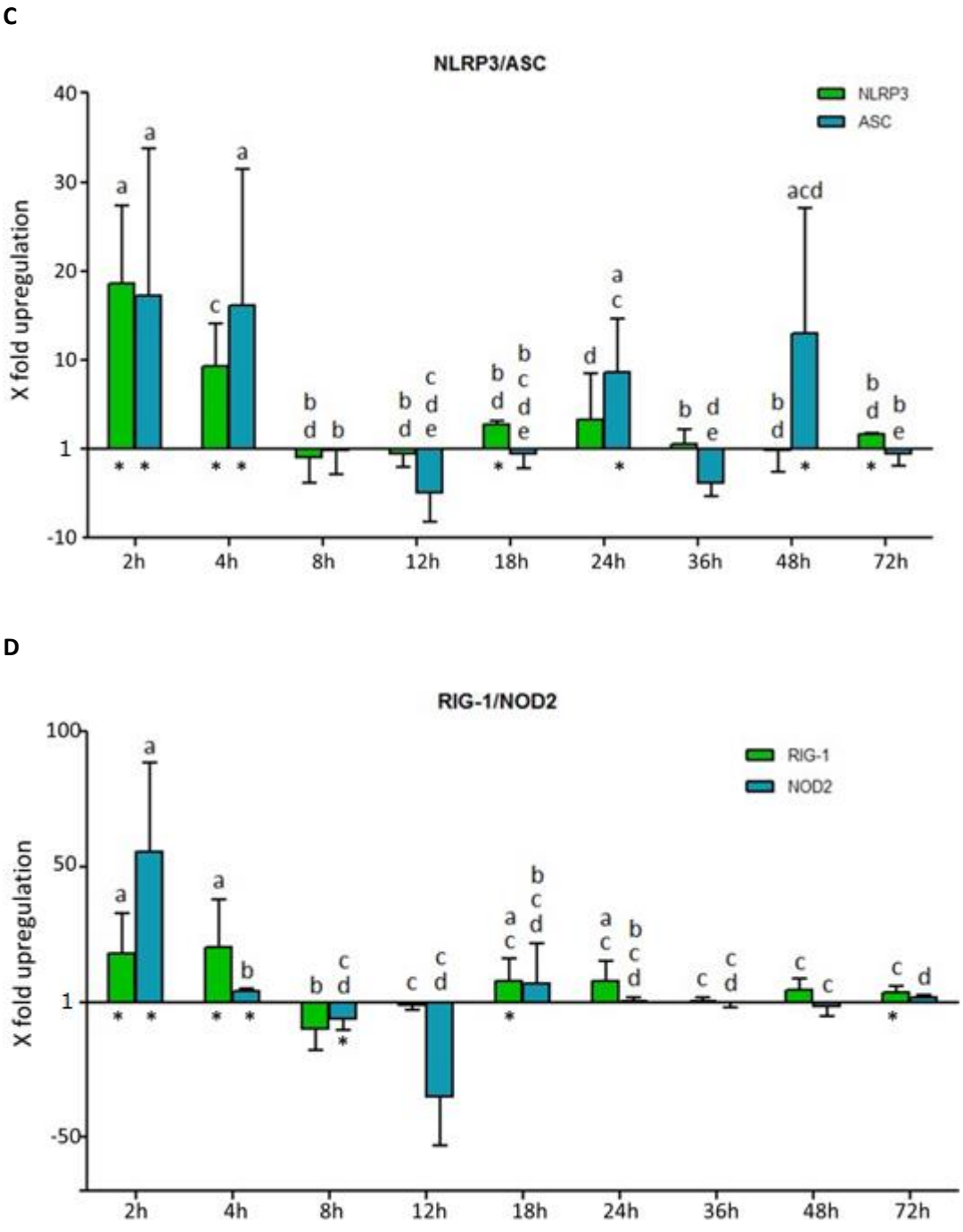
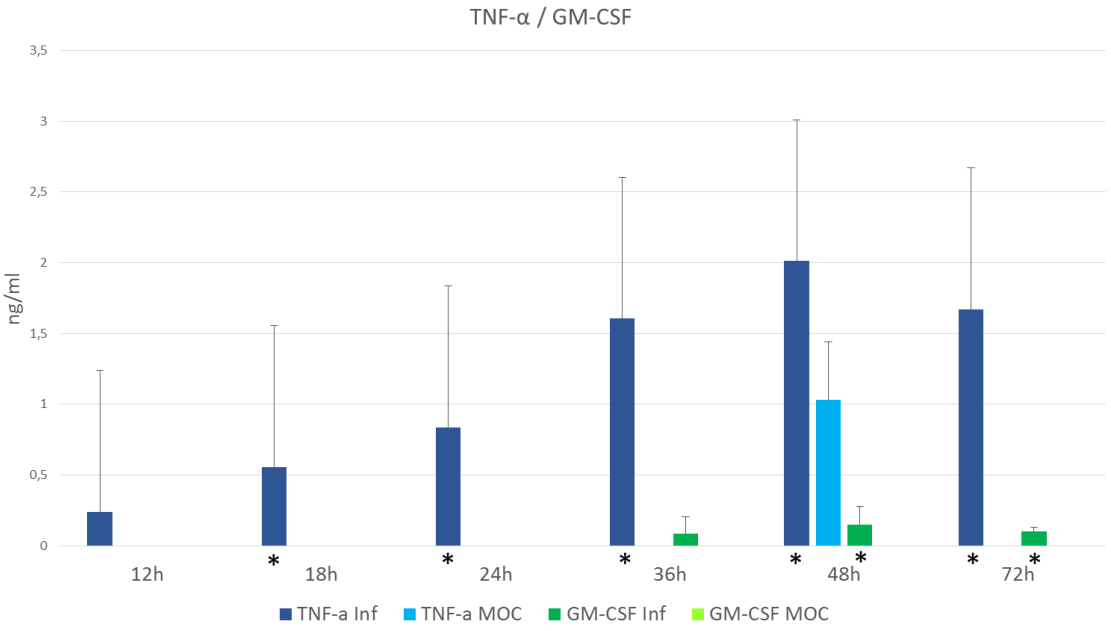


Fig. IV-8: Transcript level gene expression of TLRs/RIG-I/NODs and inflammasome by THP1 cells infected with *C. psittaci* (MOI=1) at different time points (2h, 4h, 8h, 12h, 18h, 24h, 36h, 48h, 72h) p.i. Results are presented as fold changes in mRNA levels compared to mock infected controls. Significant upregulation between the different time points for every gene, determined by an ANOVA test, is indicated by a letter ($p<0.05$). Significant differences between *C. psittaci* infected and mock infected HD11 cells, determined by a non-parametric Mann-Whitney test, are indicated by $^{*}P<0.05$. Error bars in all figures represent the standard error mean between three independent experiments performed in duplicate.

3.3. Protein analysis of human cytokines and chemokines

The concentration of different cytokines and chemokines such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IFN-γ, TNF-α and GM-CSF were determined in the supernatant of *C. psittaci* infected (MOI=1) and mock infected THP1 cells at 12h, 18h, 24h, 48h, 36h and 72h p.i.

The cytokines IL-1α, IL-2, IL-4, IL-10, IL-12, IL-17α and IFNγ were not detected in the supernatant of infected cells, indicating that those cytokines were not expressed during infection with *C. psittaci* in human macrophages. In addition, high concentrations of the cytokines IL-1β and TNF-α and the chemokine IL-8 was noticed throughout all time points measured. Remarkably, mock-infected THP1 cells also express the chemokine IL-8, indicating that there is a certain base level expression of this chemokine. The expression of the cytokine IL-6 and GM-CSF was rather late in the infection observed from 48h p.i. and 36h p.i. on, respectively (Fig. IV-9A/B/C).



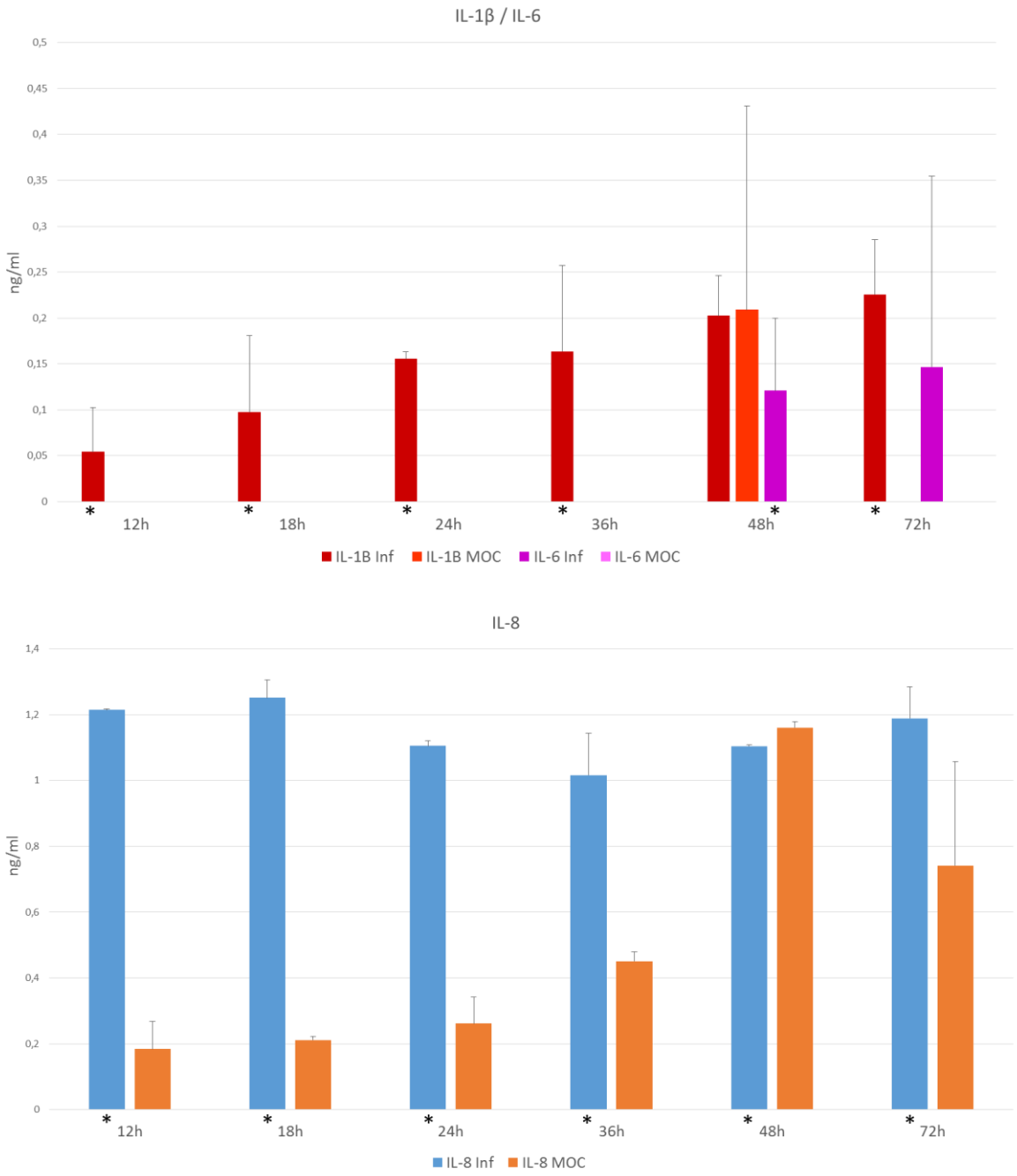


Fig. IV-9: Cytokine production of TNF-α, GM-CSF, IL-1β, IL-6 and IL-8 by THP1 cells infected with *C. psittaci* (MOI=1) at different time points (12h, 18h, 24h, 36h, 48h, 72h) p.i. Significant differences between *C. psittaci* (inf) and mock-infected (MOC) HD11 cells, determined by a non-parametric Mann-Whitney test, are indicated by * P<0.05. Error bars in all figures represent the standard deviation between three independent experiments.

4. Discussion

As mentioned before, *C. psittaci* is endemic in the poultry industry and possesses a huge zoonotic danger for poultry workers, veterinarians, employees in poultry slaughtering, laboratory workers, workers in avian quarantine stations, wildlife rehabilitators, zoo workers and pet shop workers (Harkinezhad et al., 2007; Vanrompay et al., 2007). A better understanding of the pathogenesis and the mechanisms associated with immune responses to *C. psittaci* is important to develop efficient therapeutic strategies.

It has been demonstrated in experimental infected turkeys that *C. psittaci* infect eptithelial cells and macrophages of the respiratory tract, thereby inducing severe inflammation. Macrophages are used as a transport vehicle to spread to several organs throughout the body to establish a systemic infection as *C. psittaci* has been found in blood monocytes/macrophages recognized by the specific marker KUL01 (Vanrompay et al., 1995b). Because it is important to perform chlamydial research in a matched host cell system (Roshick et al., 2006), the human monocyte cell line THP1 was used to elucidate the interaction between *C. psittaci* and its human host.

Similary with the study of Beeckman et al. (2010), a limited replication, or barely survival of *C. psittaci* was noticed in THP1 cells for all MOIs investigated. In this study, looking at the mode of entry, *C. psittaci* induces actin recruitment and polymerization to the site of invasion within 15 min p.i. However, the observed polymerization was not very pronounced suggesting that the THP1 cells also actively has taken up the bacteria via phagocytosis. Normally, after phagocytosis, lysosomes fuses with pathogen-containing phagosomes, releasing his acidic hydrolases in the resulting phagolysosomes in order to eradicate the pathogen. But *Chlamydiaceae* have developed mechanisms to avoid this phagolysosomal fusion (Escalante-Ochoa et al., 1998). However, Beeckman and Vanrompay (2010) have demonstrated by means of transmission electron microscopy experiments that after 2h p.i. lysosomal fusion takes place for *C. psittaci* genotype B (strain CP3). It could be that the significant decrease of IFU/ml from 30 minutes until 24h p.i. observed for all MOIs is the result of lysosomal fusion. A tremendous significant increase in mean fluorescence area per inclusion is observed between 24h and 48h p.i., suggesting the similarity with the developmental cycle in epithelial cells. Moreover, the microscopic images showed that *chlamydiae* were also released after 48h p.i. Interestingly, when a significant difference was observed at a certain time point between different MOIs then the smallest MOI value yields the largest mean fluorescence area per inclusion. These observations suggest that the bacteria are able to sense how many EBs are present in the neighborhood. This inter-bacterial communication is called quorum sensing which is used for cell-to-cell communication and as a sensor for population density (Bassler, 2002). Up to now, there is no indication that *Chlamydiae* is able to

sense or to communicate with other *Chlamydiae* in the inclusion. However, it is easy to imagine that detecting the number of other *Chlamydiae* bacteria or particular forms of *Chlamydiae*, especially the converting of RBs into EBs, inside the chlamydial inclusion might be useful. It could be that if *C. psittaci* senses that sufficient EBs are present, the inclusion is kept small in order to avoid disturbing the natur al host dynamics and subsequently consequent detection which result in host immune response in an attempt to eradicate the bacteria. In addition, recent studies have demonstrated that bacteria are able to control their degree of infection in order to prevent extensive activation of the immune host cell defense (Shames et al., 2010; Medzhitov, 2009). However, as *Chlamydiae* is released after 48h p.i. from THP1 cells, it is possible that the observed decrease in mean fluorescnece area per inclusion is the result of lysis of the host cell.

As the *C. psittaci* replication is limited within macrophages, man could wonder why *Chlamydiae* infect these cells in the first place. Of course, macrophages can actively take up the EBs by pahgocytosis, but the presence of the actin recruitment to the site of invasion showed that the bacteria forced entry by the use of its T3SS. Former studies demonstrate that *C. pneumoniae* also infect macrophages to transmigrate through the mucosal barrier present in the lungs and subsequently have access to the lymphatic system and the systemic circulation to spread to the other tissues present throughout the body (Moazed et al., 1998; Gieffers et al., 2004). As *C. psittaci* was already demonstrated in blood monocytes/macrophages of turkeys (Vanrompay et al., 1995b), it is suggested that *C. psittaci* also uses these cells as a vector to disseminate by hematogenous and lymphatic routes and establish a systemic infection.

At 15 min p.i., several proteins of the T3SS such as tarp, IncA and SctW were detected, which supports the hypothesis that *C. psittaci* have a preloaded T3SS. SctW – an effector soluble protein blocking the transmembrane channel – is present at 15 min p.i. with is in concordance with the observations of Beeckman et al. (2008) who detected also SctW of *C. psittaci* at 15 min p.i in HD11 cells. From 1h to 8h p.i., the effector protein SctW remains undetectable which is logical as EBs are converted to RBs and the inclusion start the develop, no blocking of the T3SS by SctW is needed. At 24h and 48h p.i., SctW re-emerges, indicating the asynchronous RB to EB differentiation and the arming of the T3SS of EBs with effector proteins in order to ensure rapid entry of the host cell. The staining data indicate that the T3SS is continuously expressed and active during the replication cycle in THP1 cells. However, a limited number of T3SS proteins were investigated at a relatively small number of time points p.i. In order to obtain a more accurate view on the role of the T3SS of *C. psittaci* in human macrophages more robust and accurate techniques will be necessary such as electron microscopy along with an overview of the complete replication cycle using live cell imaging to capture the developmental cycle in real time.

Little is known on how *C. psittaci* is recognized by its host cell, the macrophage. In this study, intracellular and membrane-bound PRRs were investigated at several time points p.i. at a MOI of 1. Looking at the membrane-bound TLRs investigated, a significant upregulation of TLR1, TLR4 and TLR6 was noticed early in the infection followed by a significant decrease during the mid- en late-cycle of *C. psittaci*. Remarkably, no significant upregulation for TLR2 on the membrane of THP1 cells was detected early in the infection with *C. psittaci*. Commonly, TLR2 and TLR4 are known as the primary PRRs involved in the detection of Gram-positive and Gram-negative bacteria respectively. However, as TLR2 is involved in the recognition of a broad range of microbial products, including peptidoglycan from Gram-positive bacteria (Takeuchi et al., 1999), bacterial lipoproteins (Brightbill et al., 1999), mycobacterial cell wall lipoarabinomannan (Means et al., 1999) and yeast cell walls (Underhill et al., 1999), it is unique that several reports indicate a critical role for TLR2 for bacterial clearance and survival during an infection with *Chlamydia pneumoniae* in macrophages (Shimada et al., 2012; Beckett et al., 2012; Netea et al., 2002; Bas et al., 2008; Agrawal et al., 2011, Darville et al., 2003). The observations of Netea et al. (2002) showed the production of the pro-inflammatory cytokines TNFα and IL-1β, but not of the anti-inflammatory cytokine IL-10, through TLR-2 dependent mechanisms. These observations were confirmed by Agrawal et al. (2011), who demonstrated an inflammatory and thus pathogenic role for TLR2. Not only the cytokines TNFα and IL-1β are upregulated through TLR2, but also IL-6 and IFN-γ. In addition, TLR4 is responsible for the production of IL-12 in response to infection with *Chlamydiae* and in the absence of a functional TLR4, IL-12 secretion is attenuated. These data suggest that TLR4 plays an important role in the initiation of the innate immune response and maintains a balance between pro- and anti-inflammatory responses (Prebeck et al., 2001; Agrawal et al., 2011). Thus, it most likely that TLR2 and TLR4 are both important to orchestrate cytokine and chemokine responses and host defense against *Chlamydiae* infections. However, several reports also indicate that TLR4 also has no role for the effective host defense against *Chlamydiae* infections (Netea et al., 2002; Beckett et al., 2012). In conclusion, it has been shown that whole bacteria or Chlamydia Hsp60 induce TLR-mediated activation, but signaling receptor differed among studies from both TLR2 and TLR4 (Vabulas et al., 2001), only TLR4 (Sasu et al., 2001; Bulut et al., 2002), only TLR2 (Netea et al., 2002), to largely TLR2 and to a minor extent TLR4 (Prebeck et al., 2001). In our study, TLR4 was significant upregulated early during the infection but no IL-12 was detected in the supernatans of the infected cultured THP1 cells. Other cytokines such as IL-1β and IL-6 were also produced after infection but not through the TLR2, suggesting that other PRRs are responsible for the induction of this pro-inflammatory cytokines. Remarkably, the anti-inflammatory cytokine IL-10 was not detected, which is in concordance with earlier mRNA results of *C. psittaci* infected avian HD11 cells (Lagae and Vanrompay, 2015). A study of Bas et al. (2008) demonstrated that MIP (Macrophage Infectivity Potentiator) –a bacterial lipoprotein exposed at the surface of EBs- was able to induce TNF-α and IL-8

secretion through the signaling pathways of TLR2/TLR1/TLR6 with the help of CD14. Our data showed the significant upregulation of TLR1 and TLR6 but not TLR2 after infection with *C. psittaci* in THP1 cells. As TLR1 and TLR6 need to form a heterodimer with TLR2 to be functional, it is clear that the observed TNF-α and IL-8 production is not derived from those signaling receptors. The cytokine TNF-α was constantly secreted at the time points p.i. investigated, which is in concordance with the results of Shemer-Avni et al. (1989) who observed secretion of TNF-α after infection of Mono Mac 6 cells (human monocytic cell line) with *C. pneumonia*, peaking at 8h p.i.

Not only the membrane-bound TLRs, but also intracellular PRRs such as TLR3, TLR9, NLRP3, NOD2 and RIG-I were investigated during this study after infection with *C. psittaci* in THP1 cells. Remarkably, all intracellular PRRs investigated were significant upregulated early in the infection (2h and 4h p.i.). The intracellular TLR9 recognize bacterial DNA containing unmethylated CpG motifs and is significant upregulated at 2h, 4h and 18h p.i. The early upregulation of TLR9 suggest the presence of chlamydial CpG DNA in the endosomes early after inoculation, supporting the hypothesis that *Chlamydiae* is also phagocytosed by macrophages followed by lysosomal fusion. These results are in concordance with the results described in the study of Lagae and Vanrompay (2015) where TLR21 – a homolog of TLR9- is significantly upregulated in avian HD11 cells after infection with *C. psittaci*. It is quite remarkable that TLR3 is also significant upregulated after infection with *C. psittaci* as TLR3 recognize viral dsRNA. However, a recent study of Derbigny et al. (2010) demonstrated that oviduct epithelial cells infected with *C. muridarium* secreted IFN-β in a TLR3-dependent manner. But the observations of our study and the study of the research group of Derbigny could not be readily explained by the current understanding of the TLR3 biology. As ssDNA or dsDNA could not stimulate TLR3, we should perhaps focused on identifying the Chlamydia TLR3 PAMP which would represent a significant contribution to a better understanding of the TLR3 biology. Possibilities could be a nonnucleic acid *Chlamydia* component or it could be that after infection with *Chlamydiae* a cellular response is induced resulting in the generation of cellular dsRNA.

The inflammasome containing caspase-1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and a nucleotide-binding oligomerization domain-like protein such as NLRP3 was investigated during a *C. psittaci* infection. The NLRP3/ASC complex was significant upregulated at 2h, 4h and 24h p.i., suggesting a role for the inflammasomse for the production of mature IL-1β during infection with *C. psittaci* in THP1 cells. IL-1β production is a thightly regulated process that requires two distinct signals: 1) a pro-inflammatory signal that leads to NF-κB activation an synthesis of pro-IL-1β and 2) activation of caspase-1 via the inflammasome that cleaves pro-IL-1β into its biologically active form. Our findings demonstrate that *C. psittaci* infection of THP1 cells leads to caspase-1 activation and IL-1β secretion through a process requiring the NLRP3 inflammasome. We can

hypothese that the first signal could be derived from activation of the TLR4 to generate pro-IL-1β. These observations were also demonstrated for *C. trachomatis*, *C. pneumoniae* and *C. muridarium* (Abdul-Sater et al., 2010; He et al., 2010).

Chapter V

Examination of the *in vivo* immune response elicited by *Chlamydia psittaci* in chickens

Lagae S., Dumont A., and Vanrompay D. Examination of the *in vivo* immune response elicited by *Chlamydia psittaci* in chickens. Veterinary Immunology and Immunopathology. In Press.

Abstract

It has since long been reported that *Chlamydia psittaci* is endemic in the poultry industry in Belgium as well as in other European countries. Nowadays, expensive antibiotic treatments are necessary to reduce mortality rate but this can lead to antibiotic resistance. Moreover, *C. psittaci* can easily be transmitted from birds to humans through the inhalation of pathogen-containing aerosols derived from faeces and eye and nostril secretions. Therefore, the need for an efficient vaccine against *C. psittaci* is augmenting. However, more research is needed to develop such a vaccine. Knowledge on the immune mechanisms of *C. psittaci* infections is crucial to understand the pathogenesis of, and immunity to this zoonotic pathogen and to act as a basis for vaccination studies. This study has investigated the *in vivo* immune response evoked by *C. psittaci* in his natural host, the chicken. Excretion of *C. psittaci*, chlamydial antibody detection in sera, blood immune cells and the mRNA expression levels of different cytokines, chemokines and one Toll-like receptor were investigated in different organs (conchae, lungs, airsacs, harderian gland, bursa fabricius and spleen) at different time points post infection (6h, 24h, 48h, 4d, 6d, 8d, 10d, 14d and 21d). A higher frequency of cytotoxic $CD8^+$ T cells and monocytes/macrophages expressing the MHC II molecule were observed in the infected group. Several cytokines and chemokines are significantly upregulated during infection but remarkably also significantly downregulated, especially at late time points. Furthermore, the only Toll-like receptor investigated, TLR4, was also significant upregulated in several organs. This study can contribute on the elucidation on how *C. psittaci* interact with his host, leading to the developing of targets for effective vaccination and therapeutic strategies for infection.

1. Introduction

Chlamydia (*C.*) *psittaci* causes respiratory infections in poultry. Transmission of this pathogen occurs through inhalation of Chlamydia-bearing dust particles and aerosol from faeces and respiratory, nostril and eye excretions. After inhalation, primary replication takes place in the epithelial cells of the upper respiratory tract. Following this initial replication, the infection spreads to epithelial cells and macrophages of the lower respiratory tract. Subsequently, the bacteria appear in blood plasma and monocytes leading to a systemic infection affecting various tissues throughout the body. Symptoms vary from inapparent to severe, depending on the chlamydial strain, stress and condition of the avian host (Vanrompay et al., 1995a).

The pathology of chicken-derived *C. psittaci* strains in SPF chickens has been examined by Yin et al. (2013a). Symptoms started at 2 days post infection and included anorexia, conjunctivitis, rhinitis, dyspnoea and intermittent diarrhea. Macroscopic lesions, observed at necroscopy, showed congestion of the lungs with grey inflammatory foci, conjunctivitis, sinusitis, rhinitis, pneumoniae, air sacculitis, pericarditis, enlargement of the spleen and liver, inflammation of the gastro-intestinal tract and congestion of kidneys and reproductive organs (Yin et al., 2013a).

However, little is known about the interaction with the host in terms of the immune response. Former studies on chlamydial immunology mainly focused on adaptive immunity against *C. trachomatis*, *C. muridarum*, *C. pneumoniae*, and *C. caviae*, whereas it is more and more clear that the very early innate immune response influences migration and activation of immune cells (Germain, 2004), so directing the adaptive immune response. Very few studies have investigated the innate immune system of the avian respiratory tract (Ariaans et al., 2008; Sarmento et al., 2008; Wang et al., 2006) and only two studies have examined innate immunity to *C. psittaci* in its natural host cell, the avian macrophage (Beeckman et al., 2010; Lagae et al., 2014). Those two studies showed a significant upregulation of mRNA expression of IL-1β, IL-6, LITAF (lipopolysaccharide-induced tumor necrosis alpha factor), IL-12p35, caspase-1, MIF, IL-10, CXCLi1, CXCLi2, CCLi3, TLR-4 and TLR-21.

Interestingly, a recent study by Kalmar et al. (2015), investigated the immune response in SPF chickens at 1, 3, 7 and 14 days post infection (dpi) after aerosol infection with a non-avian *C. psittaci* strain (DC15 isolated from an aborted calve foetus) or with *C. abortus* (sheep strain S26/3). They investigated the expression of immune related genes in the thoracic airsac at 1, 3, 7 and 14 days post infection. Their results showed that the stronger replication of the non-avian *C. psittaci* strain also evoked a more intense immune response than in the case of a *C. abortus* infection, as IFN-γ, IL-1β, IL-6, IL-17, IL-22, LITAF and iNOS genes were significantly stronger upregulated in *C. psittaci* infected animals as

compared to *C. abortus* infected chickens. Moreover, antibody responses and lymphocyte proliferative responses were significantly higher in *C. psittaci* infected chickens.

However, it would be interesting to use an avian *C. psittaci* strain and to examine the expression of immune related genes at additional (earlier and later) time points during infection in its natural host. Moreover, sampling tissues of the upper and lower respiratory tract and of additional immune tissues throughout the body of the chickens could provide additional knowledge. Therefore, this study was designed to investigate the innate and adaptive immune response in SPF chickens (*Gallus domesticus*) towards the highly virulent avian *C. psittaci* genotype D strain 92/1293. The expression of the following cytokine genes (IL-1β, IL-12p35, IL-6, LITAF, IL-10, Caspase-1) and chemokines genes (CXCLi2, CCLi3) were examined, as well as the expression of toll-like receptor 4 (TLR4). Gene expression was examined at early and late points during the infection focusing at several immune and non-immune tissues throughout the body. In addition, *C. psittaci* antibody responses were examined as well as peripheral blood mononuclear cell (PBMC) phenotypes at different time points following infection.

2. Materials and Methods

2.1. Chlamydia psittaci

The *C. psittaci* genotype D strain 92/1293, used in this study, was isolated from a pooled homogenate of the lung, a cloacal swab and the spleen of a diseased turkey. The strain originated from a severe outbreak of respiratory disease on a commercial turkey broiler farm in The Netherlands (Vanrompay et al., 1993). Bacteria were grown in Buffalo Green Monkey (BGM) cells as previously described (Vanrompay et al., 1992) and the 50% tissue culture infective dose ($TCID_{50}$) was determined by the method of Spearman and Kaerber (Mayr et al., 1974).

2.2. Study Concept

Experiments were performed in negative pressure isolators (IM 1500, Montair, Sevenum, The Netherlands). The experimental design (Table V-1) was evaluated and approved by the Ethical Committee for Animal Experiments of Ghent University (EC 2010/054). Seventy-two Specific Pathogen Free (SPF) chickens (VALO BioMedia GmbH, Germany) were randomly divided into two groups. At one week of age, group 1 was aerosol infected during one hour using a Cirrus™ nebulizer (5 µm aerosol particle size, Laméris, Aartselaar, Belgium). Chickens were infected with 10^6 $TCID_{50}$ of the *C. psittaci* genotype D strain 92/1293 in phosphate buffered saline (PBS). Group 2 was aerosol mock-infected using PBS.

Eight chickens of each group were sequentially euthanized at 6 hours (h), 24h, 48h, 4 days (d), 6d, 8d, 10d, 14d and 21d post infection (p.i.). Before euthanasia, blood was taken. Sera were examined for the presence of *C. psittaci* antibodies using a recombinant major outer membrane protein MOMP-based ELISA (Verminnen et al., 2006). During necropsy, the conchae, lungs, thoracic and abdominal airsacs, harderian gland, bursa Fabricius and spleen were collected for gene expression analysis. Tissues were snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. Pharyngeal and cloacal excretion of viable *C. psittaci* was also monitored at euthanasia. Sampling was performed using rayon-tipped, aluminium shafted swabs (Copan, Fiers, Kuurne, Belgium) provided with transport medium (Vanrompay et al., 1992). Swabs were stored at -80°C until examined by culture in BGM cells. At 7d, 14d and 21d p.i., heparinized peripheral blood samples were collected for flow cytometric identification of monomorphonuclear cells.

Table V-1: Experimental set-up

Group	**7 days of age inoculation with**	**Observations, manipulations, sampling**			
		Pharyngeal + cloacal swabs 8 birds [dpi][a]	Blood 8 birds [dpi][b]	Blood 8 birds [dpi][c]	Euthanasia 8 birds [dpi][d]
1 (n = 36)	*C. psittaci* 92/1293	⅓, 24, 48, 4, 6, 8, 10, 14, 21	⅓, 24, 48, 4, 6, 8, 10, 14, 21	7, 14, 21	⅓, 24, 48, 4, 6, 8, 10, 14, 21
2 (n = 36)	PBS	⅓, 24, 48, 4, 6, 8, 10, 14, 21	⅓, 24, 48, 4, 6, 8, 10, 14, 21	7, 14, 21	⅓, 24, 48, 4, 6, 8, 10, 14, 21

[a]: Chlamydia cell culture [b]: Antibody determination (ELISA) [c]: Flow cytometric phenotyping of PBMCs [d]: Quantitative real-time PCR of different organs

2.3. *Chlamydia psittaci excretion*

Swabs were shaken for 1 h at 4°C, centrifuged and *Chlamydia* excretion was monitored using standard procedures for culture and bacterial identification (IMAGEN™ immunofluorescence staining kit; ThermoFisher, Aalst, Belgium). The presence of *C. psittaci* on the whole microscopic slide was enumerated (600x, BX41 Olympus, Aartselaar, Belgium) and results were scored from 0 to 8. (Table V-2).

Table V-2: Culture scores

Score	Meaning
0	No EB's or inclusions
1	1-3 EBs
2	3-10 EBs and 1-2 IPCs
3	10-30 EBs and 3-10 IPCs
4	30-50 EBs and 11-20 IPCs
5	50-70 EBs and 21-30 IPCs
6	70-100 EBs and 31-50 IPCs
7	> 100 EBs and 51-70 IPCs
8	All of the fields EBs and IPCs

EB = elementary body, IPC = Inclusion with replicating *C. psittaci*

2.4. Flow cytometric phenotyping of PBMC

Peripheral blood mononuclear cells (PMBC) to be used for flow cytometric phenotyping were purified from heparinized peripheral blood (*v. ulnaris*) by lymphoprep density gradient centrifugation. PBMC were cryopreserved in liquid nitrogen until analysis.

For analysis, PBMC were washed and resuspended in staining medium consisting of RPMI 1640 supplemented with 1% bovine serum albumin (BSA) and 0,1% NaN_3. PBMC were stained using the following monoclonal antibodies (Southern Biotech, Birmingham, AL): *Panel 1*: CD4-RPE, CD3-SPRD, CD8α-Cy5, TCR1-biotin (TCRγδ), streptavidin-APC-Cy7. *Panel 2:* CD45-FITC, CD44-APC. *Panel 3*: KUL01-FITC, MHC Class II-PE. Staining was done by adding the monoclonal antibodies in combination or as single-color staining for compensation to 3 x 10^5 cells. Cells were incubated at 4°C for 30 min and washed three times with washing buffer. Subsequently (only panel 1) streptavidin-APC-Cy7 was added and cells were incubated at 4°C for 30 min. Cells were washed three times with washing buffer. All flow cytometric analyses were conducted using a BD FACSCanto™ (Beckton Dickinson, Erembodegem, Belgium) equipped with a 488 nm blue laser and a 633 nm red laser. A minimum of 40 000 lymphocytes were collected from each sample and analyzed using the FACSDiva software.

2.5. Chlamydial antibody detection

The sera were analyzed using a previously developed recombinant MOMP-based ELISA (Verminnen et al., 2006). Serum antibody titres were determined in 2-fold dilution series stating at a dilution of 1/40. Anti-MOMP immunoglobulin titers were determined as the highest serum dilution that gave an optical density (OD_{405}) above the cut-off value. The cut-off value was the mean of the OD values of negative chicken sera plus two times the standard deviation. Titers were presented as the reciprocal of the highest serum dilution with an absorbance above the cut-off value. Negative control sera were obtained from 1-week-old Specific Pathogen Free (SPF) chickens. Positive control sera originated from a former experimental infection (Yin et al., 2013a).

2.6. Quantitative Real-Time PCR

Frozen tissues were thawed and homogenized for total RNA extraction using TRIzol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's protocol. After RNA extraction, samples were treated with RNase-free amplification grade DNase I (Promega) following the manufacturer's instructions and were confirmed to be DNA-free by performing a PCR for the presence of the *C. psittaci* 16S rRNA gene. One microgram of total RNA was reverse transcribed (reverse-IT^{TM} 1st Strand Synthesis, Thermo Scientific) into host cell cDNA using the anchored oligo-dT molecule. Specific primers (Table V-3) were designed using primer 3 (http://frodo.wi.mit.edu/primer3/) and DINA melt (http://www.bioinfo.rpi.edu/ applications/hybrid) software programs. Specificity of all qRT-PCR primers was initially checked by conventional PCR, cloning (pGEM-T Easy Vector System, Promega, Leiden, The Netherlands) and DNA sequencing of the inserts (LGC Genomics, Berlin, Germany). As we were not able to design specific primers for IL-10 and IL-12p35, probes labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the N,N,N,N'-tetramethyl-6-carboxylrhodamine (TAMRA) quencher at the 3'end (designed by P. Kaiser and L. Rothwell; Institute for Animal Health, Compton, Berkshire, UK) were needed to verify the specificity of the amplified targets (Table V-3).

Table V-3: Real-time quantitative PCR primers and probes.

Target	Accession No.	Primer and probe sequence (5'-3')	T_a
28S rRNA	X59733	F: TTTGGGTTTTAAGCAGGAGGT R: TTGCGACAACACATCATCAGT	58°C
IL-1β	Y15006.1	F: CACAGAGATGGCGTTCGTT R: GTGACGGGCTCAAAAACCT	58°C
IL-6	NM_204628	F: AGAAATGCCTGACGAAGCTCT R: CACGGTCTTCTCCATAAACGA	58°C
Caspase-1	AF031351	F: TGCCATGAAGACAAAACTTCC R: TCTACACATCTCCAGCCATCC	58°C
IL-10	AJ621254	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG P: CGACGATTCGGCGCTGTCACC	55°C
TNF-α	AY765397	F: TCCTCACCCCTACCCTGTC R: TCAGAGCATCAACGCAAAAG	58°C
IL-12p35	AJ262751	F: TGGCCGCTGCAAACG R: ACCTCTTCAAGGGTGCACTCA P: CCAGCGTCCTCTGCTTCTGCACCTT	55°C
CXCLi2	NM_205498	F: CTCGCTCTTCTCATCGCATC R: GGCAGCAGTGTCCCATCC	58°C
CCLi3	Y18692	F: AGCCTGCCATCATCTTCATC R: AAACAGCACCTGCCATGAG	58°C
TLR4	NM_001030693	F: TGGCACCTACCCTGTCTTTC R: GGCTTGGAGTGGCTTGTATG	58°C

F: forward primer; R: reverse primer; P: 5'-FAM (5-carboxyfluorescein) + 3'-TAMRA (6-carboxytetramethylrhodamine) probe.

Following cDNA synthesis, cDNA amplification was performed for IL-1β, IL-12p35, IL-6, TNF-α, CXCLi2, CCLi3, Caspase-1, IL-10, TLR4 and the 28S rRNA normalization gene using the Absolute™ QPCR SYBR® Green Mix (Qiagen) or the Absolute QPCR mix (Thermo Scientific). RNA expression of selected genes was determined by quantitative real-time PCR (qRT-PCR) with the Rotor Gene Q (Qiagen) using the following cycle profile: one cycle of 95°C for 15 min and 45 cycles of 95°C for 30s, 55-58°C for 30s and 72°C for 30s. Quantification was performed as described by Beeckman *et al.,* (2008), using standard graphs of the cycle treshold (Ct) values obtained by testing 10-fold serial dilutions (10^9 to 10^1 molecules/µl) of the purified PCR products. Ct-values of the samples were automatically converted into initial template quantities (N_0) by use of the RotorGene software Q (Qiagen) using imported standard curves from previous runs. No difference in mRNA level is shown as a fold change of 1.

2.7. Statistical analysis

To compare between the infected and the mock-infected group, standard error means were calculated and an unpaired student's t-test was used. Secondly, an analysis of variance (ANOVA, SPSS Inc., Chicago, IL, USA) with post-hoc analysis was performed to determine significant differences ($p < 0.05$) between the different time points under study.

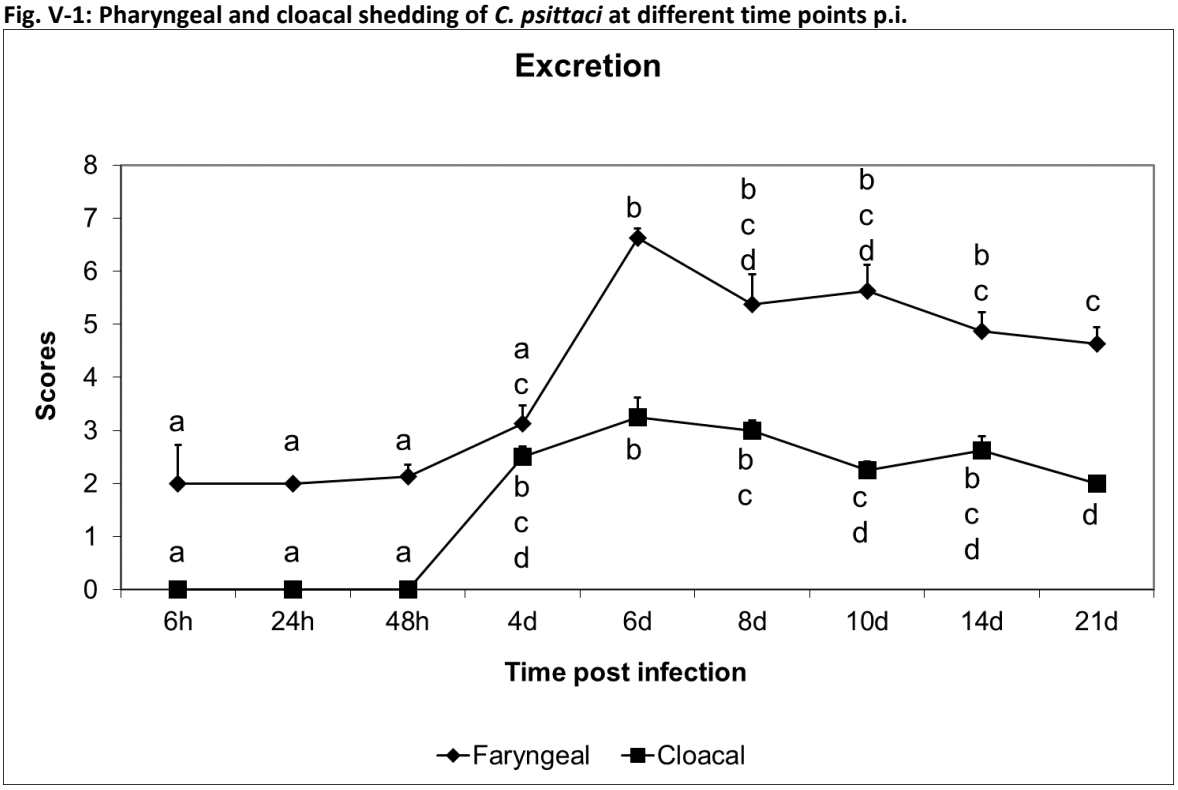
3. Results

3.1. Chlamydia psittaci pharyngeal and cloacal excretion

Cultures scores for the pharyngeal and cloacal swabs are presented in Fig V-1. Negative control chickens did not excrete *C. psittaci*. At 6h p.i. *C. psittaci* was detected in pharyngeal swabs with a mean culture score of 2. The same amount of *C. psittaci* was also present in swabs taken at 24h and 48h p.i. From 4 days post infection (dpi) onwards pharyngeal shedding significantly increased (mean culture score of 3.1) with the highest amount of *C. psittaci* found at 6 dpi (mean culture score of 6.6). From 6 dpi onwards, pharyngeal excretion gradually decreased but it remained statistically the same till 14 dpi.

Cloacal shedding of *C. psittaci* was observed from 4 dpi. onwards. Cloacal excretion was the highest at 6d p.i. (mean score of 3.2) followed by a significant decline at 10d p.i. (mean score 2.25). However, at 14 dpi, cloacal excretion again increased significantly to a level comparable to the one observed at 6 dpi. At 21 dpi, cloacal excretion was significantly lower than on all previous time points. Throughout the infection, cloacal excretion was lower than pharyngeal excretion.

Fig. V-1: Pharyngeal and cloacal shedding of *C. psittaci* at different time points p.i.



Each value represents a mean of 8 infected chickens. Significant differences between the time points post infection is indicated by a letter ($p<0.05$). The error bars represent the standard error of the mean.

3.2. Phenotyping of PBMC

Immunophenotyping of peripheral blood lymphocytes subpopulations was performed by flow cytometry using three different staining panels. When analyzing the samples stained with antibody panel 1, first the lymphocyte population was located in a FCS/SSC dot plot, next T cells were identified as being $CD3^+$. T cells were divided into γδ T cells ($TCR1^+$) and non-γδ T cells and analyzed for their expression of the surface markers CD4 and CD8α. When examining the samples stained with antibody panel 2, first the lymphocyte population was located in a FCS/SSC dot plot, next thrombocytes were excluded as being $CD45^{low}$. Subsequently, CD44 and CD45 expression was analyzed on the lymphocyte population. When analyzing samples stained with antibody panel 3, first the granulocyte and monocyte population was located in a FCS/SSC dot plot, next monocytes/macrophages were identified as being $KUL01^+$. Finally MHC Class II expression was analyzed on the monocytes/macrophages (FIg. V-2). The results of the phenotyping analyses are presented in Table V-2.

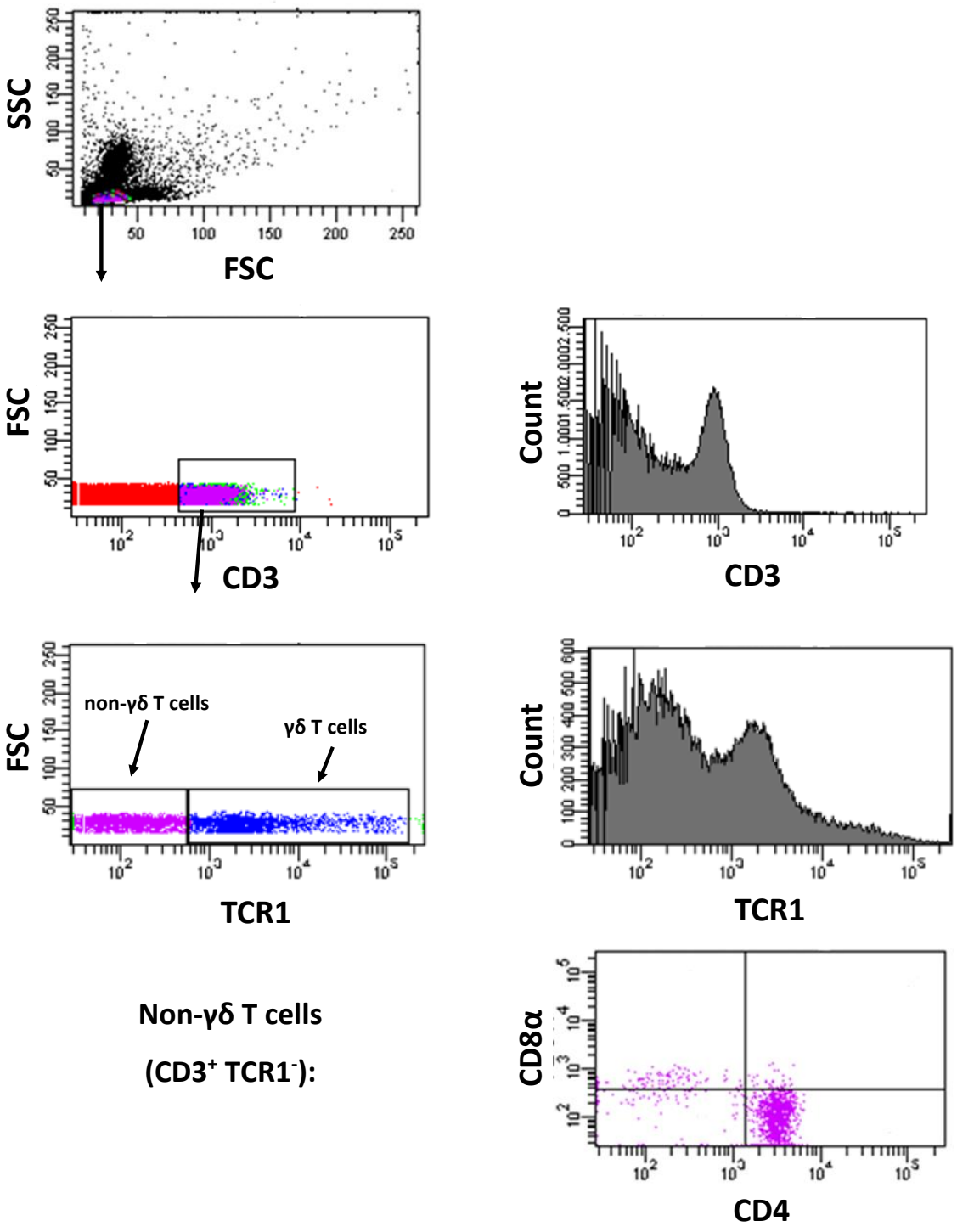
No significant differences between the percentage of non-γδ T cells of the infected and mock-infected chickens were noticed. Within the non-γδ T cell population, the frequency CD4 and CD8α cells was determined. No significant differences between the frequency of CD4 cells of the infected and mock-infected chickens were observed. However, the infected group had a significant higher frequency of CD8α cells compared with the mock-infected group at all time points p.i. investigated.

It was noticed that the percentage of γδ T cells from the infected animals was significantly higher than the values from the mock-infected controls at 7d p.i. Within the γδ T cell population the frequency of CD4 and CD8α cells was determined. No $CD4^+$ cells were observed but CD8α frequencies are presented in table V-4. No significant differences between the infected and mock-infected group were detected.

The expression of CD44 and CD45 was determined on peripheral lymphocytes ($CD45^{hi}$) and presented as mean fluorescence intensities (MFI). At 21d p.i., the mock-infected group has a significant higher expression of CD45 on their peripheral lymphocytes compared with the infected group.

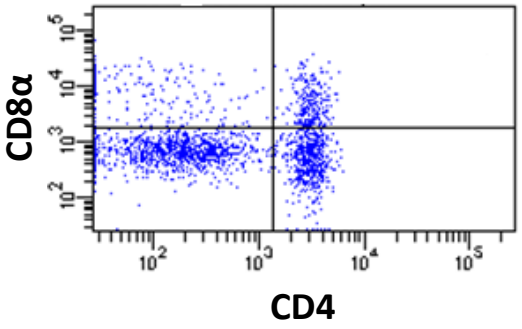
Regarding at the monocytes/macrophages, presented as being $KUL01^+$, no significant differences in the frequency between the infected and mock-infected group was observed. However, at 7d p.i. a significant higher frequency of monocytes/macrophages presenting the MHC Class II molecule on their surface was noticed in the infected group compared with the mock-infected control group. At 21d p.i., the mock-infected control group showed a significant higher percentage of MHC Class II^+ monocytes/macrophages compared with the infected group.

A

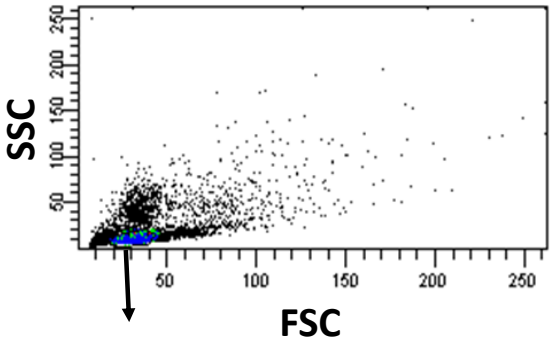

SSC
FSC
FSC
CD3
Count
CD3
non-γδ T cells
γδ T cells
FSC
TCR1
Count
TCR1
Non-γδ T cells
(CD3+ TCR1-):
CD8α
CD4

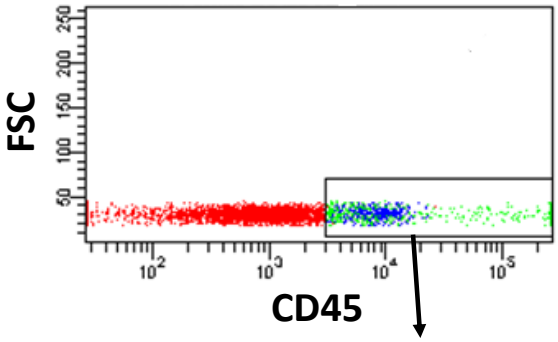
γδ T cells

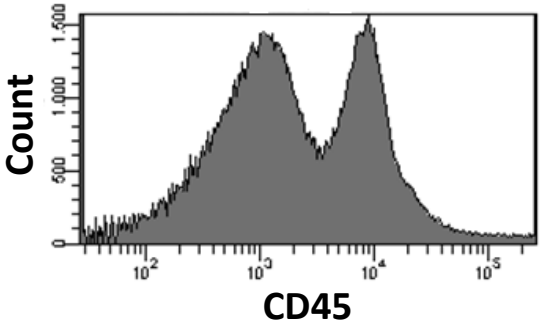
(CD3⁺ TCR1⁺):

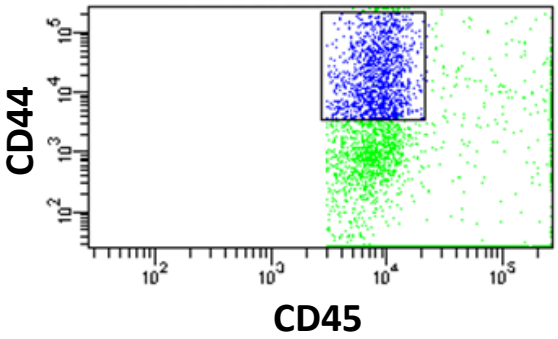


B









C

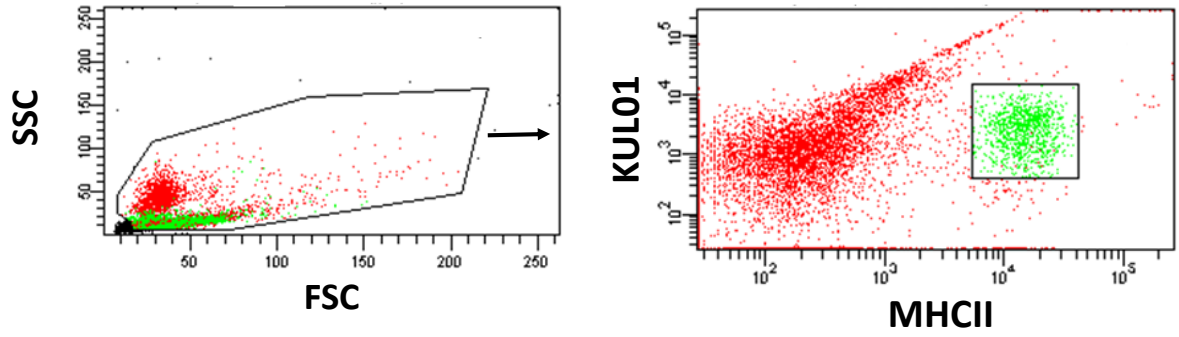


Fig. V-2: Immunophenotyping staining profiles of chicken mononuclear cells from peripheral blood. (A) A representative sample stained with antibody panel 1 (CD3, TCR1, CD4, CD8α). The staining strategy was: lymphocytes, $CD3^+$ cells, $TCR1^+$ cells. $CD3^+TCR1^+$ and $CD3^+TCR1^-$cells were analyzed for CD4 and CD8α expression. (B) A representrative sample stained with antibody panel 2 (CD45, CD44). The gating strategy was to identify the lymphocytes in a SSC/FSC plot and subsequently to exlude thrombocytes as being $CD45^{low}$. $CD45^{high}$ cells were analyzed for their CD44 expression. (C) A representative sample stained with antibody panel 3 (KUL01, MHCII). $KUL01^+$ cells were analyzed fort heir MHC Class II expression.

Table V-4: PBMC from mock-infected and *C. psittaci* infected chickens were subjected to immunophenotyping by flow cytometry at different time points (7d, 14d and 21d) p.i. Each value represents a mean of 8 chickens.

		Mean percentage ± standarddeviation of:							Mean Fluorescence Intensity ± standarddeviation of:	
PMBC [dpi]	**Group**	**$CD3^+TCR1^-$ cells***	**$CD3^+TCR1^-$ $CD4^+$ cells***	**$CD3^+TCR1^-$ $CD8\alpha^+$ cells***	**$CD3^+TCR1^+$ cells***	**$CD3^+TCR1^+CD8\alpha^+$ cells***	**$KUL01^+$ cells***	**$KUL01^+MHCII^+$ cells***	**CD44***	**CD45***
7	**A**	80.5 ± 3.8	76.1 ± 3.6	11.0 ± 1.7[b]	20.8 ± 2.7[b]	15.5 ± 4.9	19.5 ±13.5	26.4 ± 6.2[b]	104 350 ± 15630	9 746 ± 452
	B	80.5 ± 5.3	71.8 ± 10.7	4.9 ± 1.8[b]	17.0 ± 1.2[b]	18.1 ± 6.5	21.4 ±12.9	15.3 ± 3.1[b]	92 138 ± 23594	10 173 ± 493
14	**A**	77.7 ± 6.3	69.2 ± 8.3	10.5 ± 2.8[a]	22.5 ± 6.3	18.2 ± 7.7	69.2 ± 4.7	14.6 ± 4.9	128 517 ± 12 828	7 868 ± 907
	B	81.5 ± 5.9	68.7 ± 13.9	7.9 ± 1.7[a]	18.7 ± 6.1	13.8 ± 4.7	71.5 ±13.8	15.3 ± 3.0	119 533 ± 13 642	7 696 ± 1158
21	**A**	75.4 ± 4.9	76.0 ± 5.2	14.2 ± 5.7[a]	23.7 ± 4.2	3.4 ± 1.3	33.7 ± 9.8	14.3 ± 1.8[b]	80 918 ± 1 827	5 050 ± 278[a]
	B	72.4 ± 4.9	79.8 ± 3.1	8.4 ± 3.2[a]	27.6 ± 4.7	2.8 ± 0.9	33.4 ± 8.4	17.9 ± 2.7[b]	80 915 ± 4 104	5 295 ± 225[a]

* $CD3^+TCR1^-$ cells: the proportion of T cells ($CD3^+$) that does not express the γδ T cell receptor ($TCR1^-$); $CD3^+TCR1^-CD4^+$ cells: the proportion of non-γδ T cells ($CD3^+TCR1^-$) that express CD4; $CD3^+TCR1^-CD8\alpha^+$ cells: the proportion of non-γδ T cells ($CD3^+TCR1^-$) that express CD8α; $CD3^+TCR1^+$ cells: the proportion of T cells ($CD3^+$) that expresses the γδ T cell receptor ($TCR1^+$); $CD3^+TCR1^+CD8\alpha^+$ cells: the proportion of γδ T cells ($CD3^+TCR1^+$) that express CD8α; $KUL01^+$ cells: the proportion monocytes/macrophages ($KUL01^+$); $KUL01^+MHCII^+$ cells: the proportion monocytes/macrophages ($KUL01^+$) that express MHCII; CD44: the surface expression of CD44 on lymphocytes; CD45: the surface expression of CD45 on lymphocytes. [a] $P<0.05$ for a comparison of the control group and the infection group determined by an unpaired student t test. [b] $P<0.005$ for a comparison of the control group and the infection group determined by an unpaired student t test.

3.3. Serological analysis of chicken sera

Negative control chickens remained seronegative during the experiment. From 4 days p.i. onwards, low *C. psittaci* antibody titers were found in infected chickens (mean titer: 45 ± 11.8) with the highest mean antibody titer found at 6d p.i. (titer: 775 ± 313) (Fig. V-3). Between 6d p.i. and 10d p.i. (titer: 700 ± 221), a plateau phase was observed, followed by a decline of the mean antibody titer towards 21 d p.i. (titer: 55 ± 13) . Although, no statistically difference was observed between the different time points investigated.

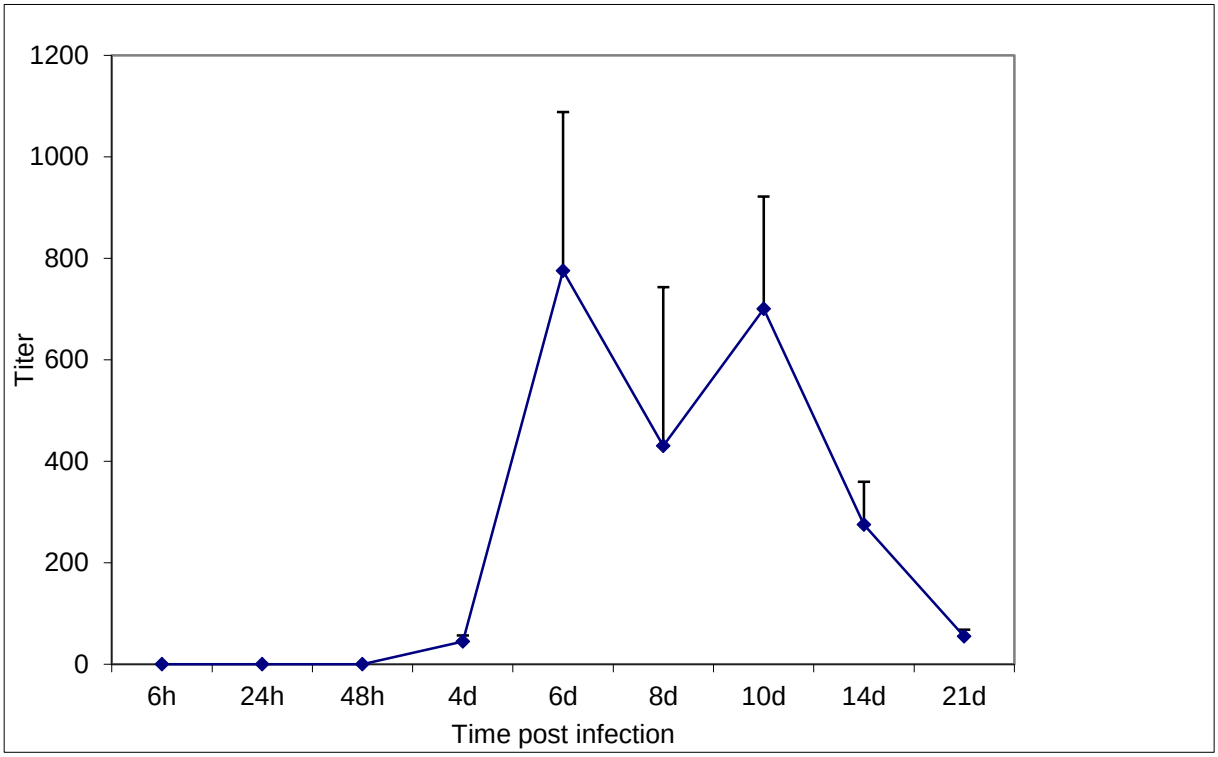


Fig. V-3: Mean MOMP-specific serum antibody titers of the infected group ± standard deviation at different time points following infection with *C. psittaci* 92/1293. Each value represents a mean of 8 infected chickens. The error bars represent the standard error of the mean.

3.4. Quantitative Real-Time PCR

Presented in figure V-3, the first initiation of the innate immune response was seen at 24h p.i. in the conchae with a significant upregulation of the pro-inflammatory genes IL-1β, TNF-α, CXCLi2, CCLi3 and caspase-1. At 48h p.i., mRNA expression levels of all genes investigated were significantly upregulated in the conchae , except for CCLi3 and IL10 whereas the latter was never significantly up- or downregulated during the infection in the conchae. (Fig. V-4).

Later in the infection, at 4d p.i. a significant upregulation in the mRNA expression levels of IL-1β, IL-12p35, TNF-α, CCLi3, caspase-1 and TLR4 were observed in the lungs whereas the expression of TLR4

reaches a significant peak at 6d p.i. Again, the expression levels of the regulatory cytokine IL-10 were unchanged during the infection in the lung tissue (Fig. V-5).

Interestingly, the airsacs, a tissue which is anatomically in close contact with the lungs, showed also a significant upregulation of the expression levels of pro-inflammatory cytokines like IL-1β, IL-12p35, IL-6, TNF-α and caspase-1 at 4d p.i., followed by a significant downregulation of IL-12p35 and IL-6 at 6d p.i. (Fig. V-6). At 14d p.i., the mRNA expression levels of caspase-1 and CXCLi2 was significantly upregulated whereas the latter gene was significantly downregulated at 21d p.i. in the airsacs. The anti-inflammatory cytokine IL-10 was significantly downregulated at 21d p.i. (Fig. V-6).

Figure V-8 shows a significant upregulation of the mRNA expression of all genes investigated at 8d, 10d and 14d p.i. in the bursa fabricius. Remarkably, TLR4 was already significantly upregulated from 48h p.i. onwards with a significant peak at 14d p.i. This peak correlates well with a significant upregulation of all the pro-inflammatory cytokines and chemokines genes investigated. Furthermore, the mRNA expression level of the anti-inflammatory cytokine IL-10 was also significantly upregulated at 10d and 14d p.i. in the bursa fabricius (Fig. V-8).

In the spleen, mRNA expression of IL-1β was not observed (data not shown). Nevertheless, all the other pro-inflammatory cytokines (IL-12p35, IL-6 and TNF-α) were significantly upregulated at 8d p.i., indicatinging that a systemic infection is established from 8d p.i. onwards (Fig. V-8). Interestingly, TLR4 was significantly very high upregulated at 6d p.i. in the spleen followed by a decline whereas the mRNA expression level remains unchanged from 14d p.i. onwards until the termination of the experiment (Fig. V-9).

This fact was also seen in the harderian gland with a significant high upregulation of TLR4 at 6d p.i. (Fig. V-7). Expression levels of IL-1β mRNA were significantly upregulated at 48h and 10d p.i. in the harderian gland which correlates well with the upregulation of caspase-1 at 48h and 8d p.i. Furthermore, a significant upregulation of TNF-α at 6d and 10d p.i., of IL-6 and IL-12p35 at 14d p.i. was observed in the harderian gland. Remarkably, no significant upregulation of the mRNA expression levels of the pro-inflammatory chemokines CXCLi2 and CCLi3 was seen, only a significant downregulation of CCLi3 at 24h p.i. Expression levels of IL-10 mRNA were significantly upregulated at 8d p.i. (Fig. V-7).

Conchae:

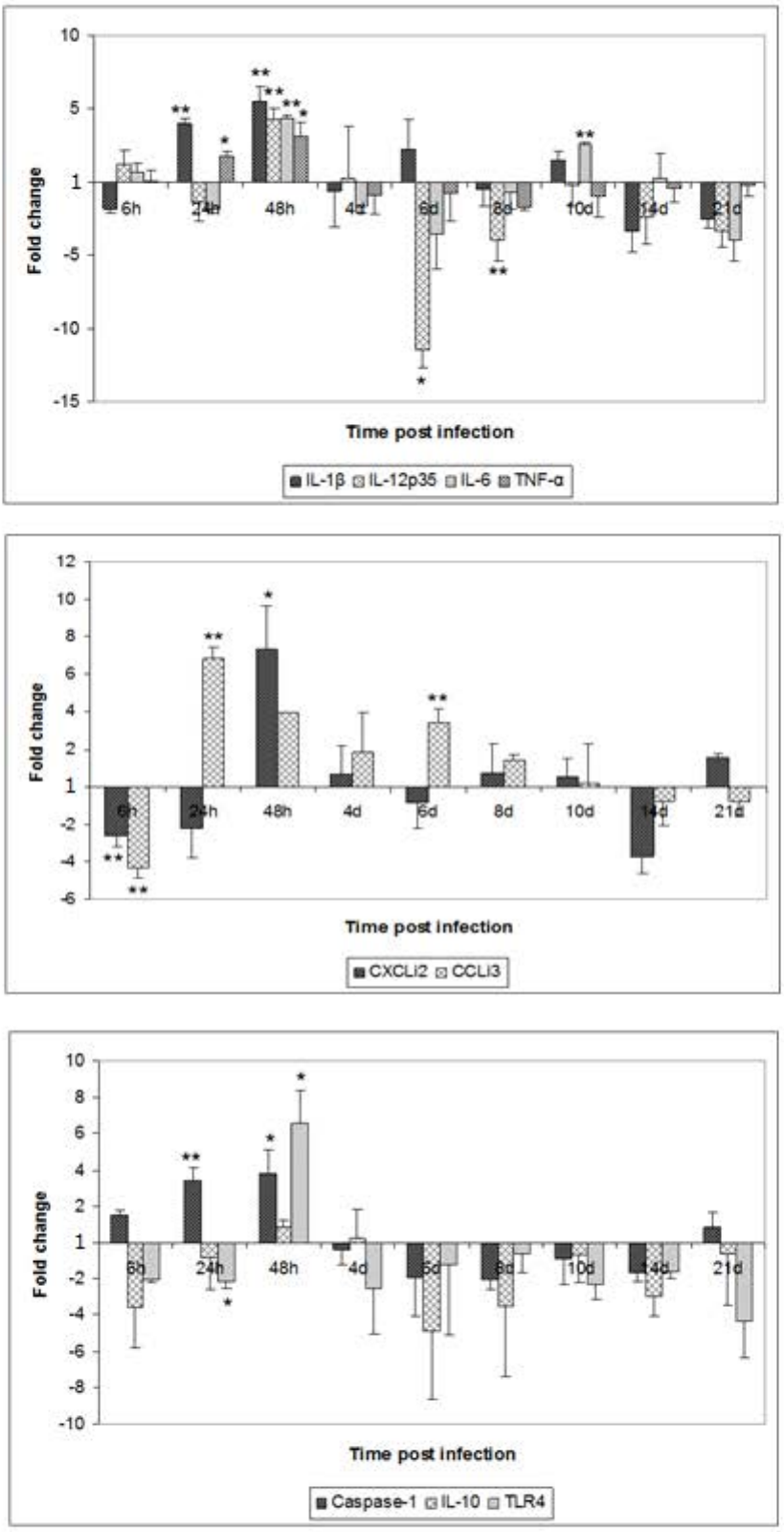


Fig. V-4: Expression of cytokine or chemokine or toll-like receptors in the conchae of chickens following infection with *C. psittaci*. Results are presented as fold changes in mRNA compared with age-matched mock-infected controls based on samples from four chickens for each time determined by quantitative RT-PCR. Significant differences between *C. psittaci* infected and mock-infected chickens, determined by an unpaired student t test, are indicated by $^{**}P< 0.05$ and $^{*}P<0.1$. The error bars in all figures represent the standard error of the means.

Lungs:

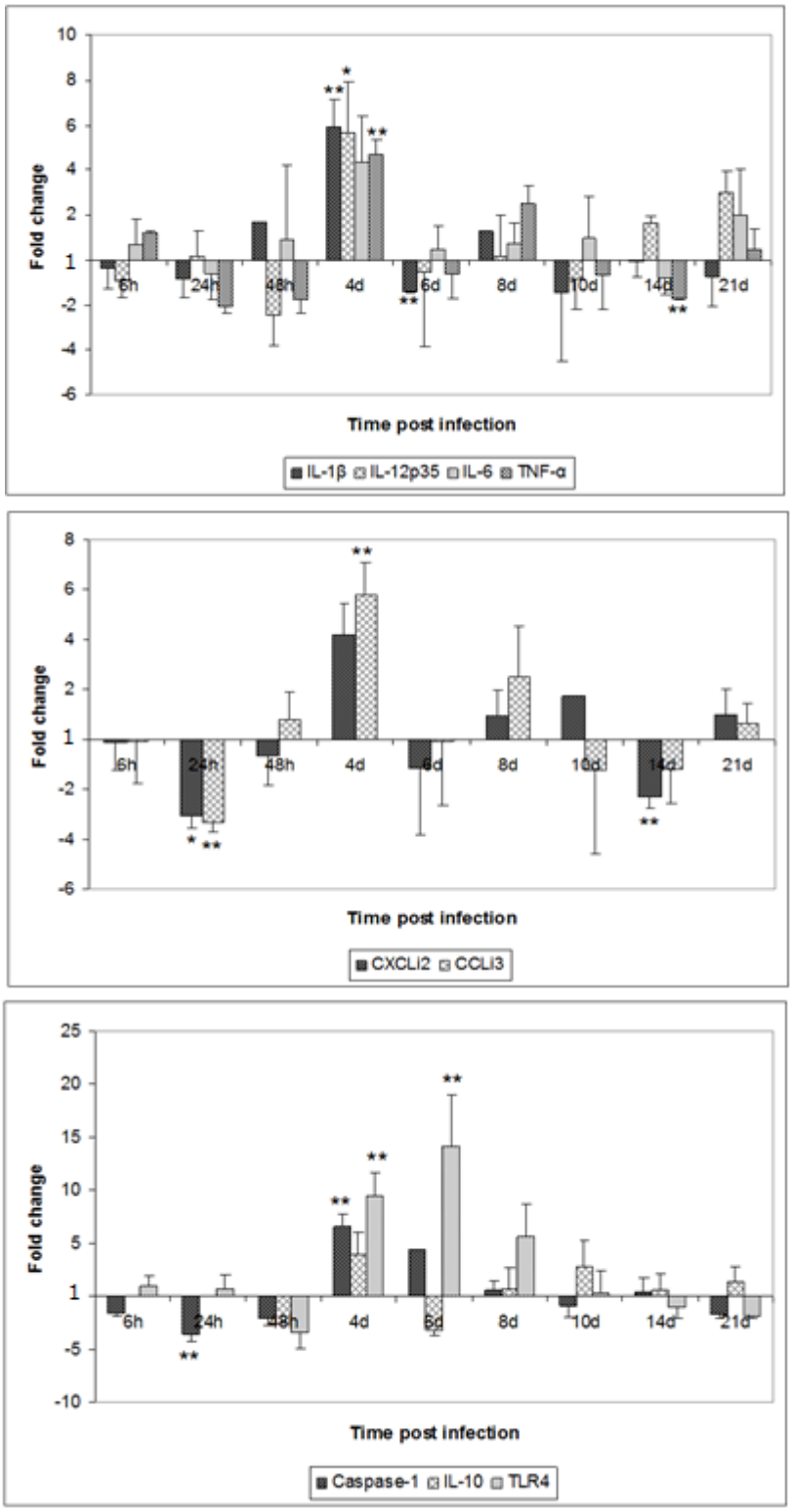


Fig. V-5: Expression of cytokine or chemokine or toll-like receptors in the lungs of chickens following infection with *C. psittaci*. Results are presented as fold changes in mRNA compared with age-matched mock-infected controls based on samples from four chickens for each time determined by quantitative RT-PCR. Significant differences between *C. psittaci* infected and mock-infected chickens, determined by an unpaired student t test, are indicated by $^{**}P< 0.05$ and $^{*}P<0.1$. The error bars in all figures represent the standard error of the means.

Airsacs:

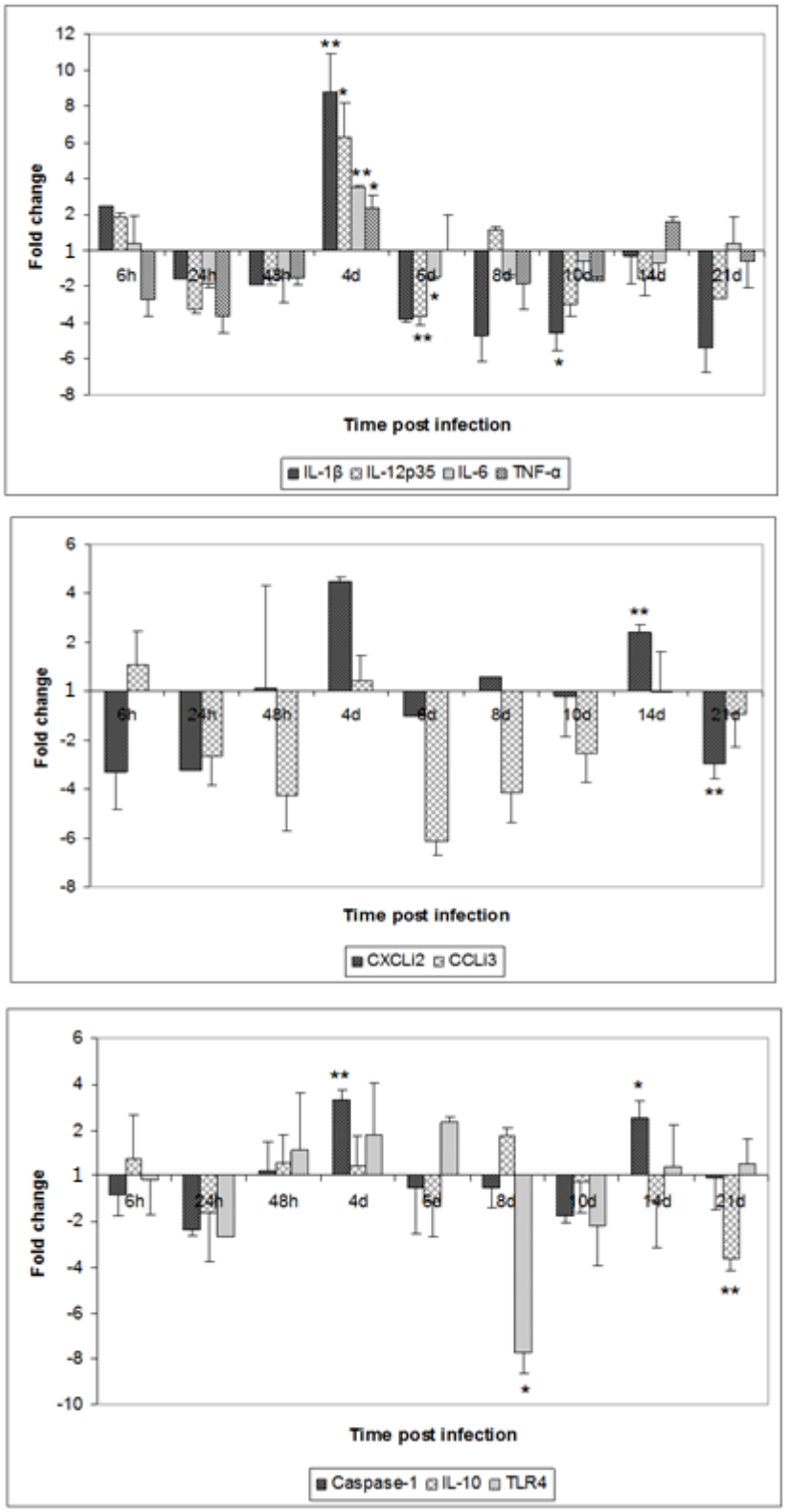


Fig. V-6: Expression of cytokine or chemokine or toll-like receptors in the airsacs of chickens following infection with *C. psittaci*. Results are presented as fold changes in mRNA compared with age-matched mock-infected controls based on samples from four chickens for each time determined by quantitative RT-PCR. Significant differences between *C. psittaci* infected and mock-infected chickens, determined by an unpaired student t test, are indicated by **P< 0.05 and *P<0.1. The error bars in all figures represent the standard error of the means.

Harderian gland:

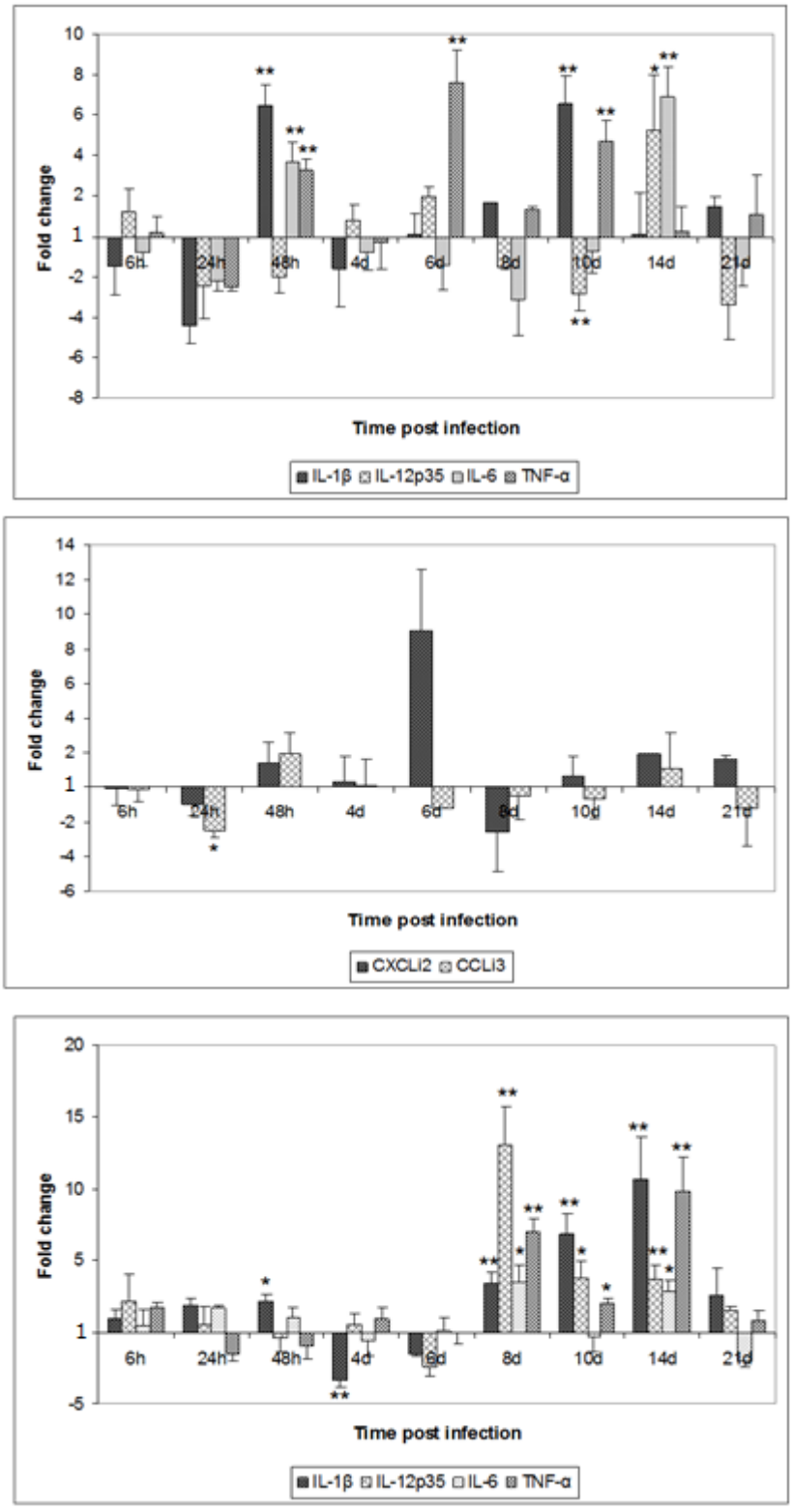


Fig. V-7: Expression of cytokine or chemokine or toll-like receptors in the harderian gland of chickens following infection with *C. psittaci*. Results are presented as fold changes in mRNA compared with age-matched mock-infected controls based on samples from four chickens for each time determined by quantitative RT-PCR. Significant differences between *C. psittaci* infected and mock-infected chickens, determined by an unpaired student t test, are indicated by $^{**}P< 0.05$ and $^{*}P<0.1$. The error bars in all figures represent the standard error of the means.

Bursa fabricius:

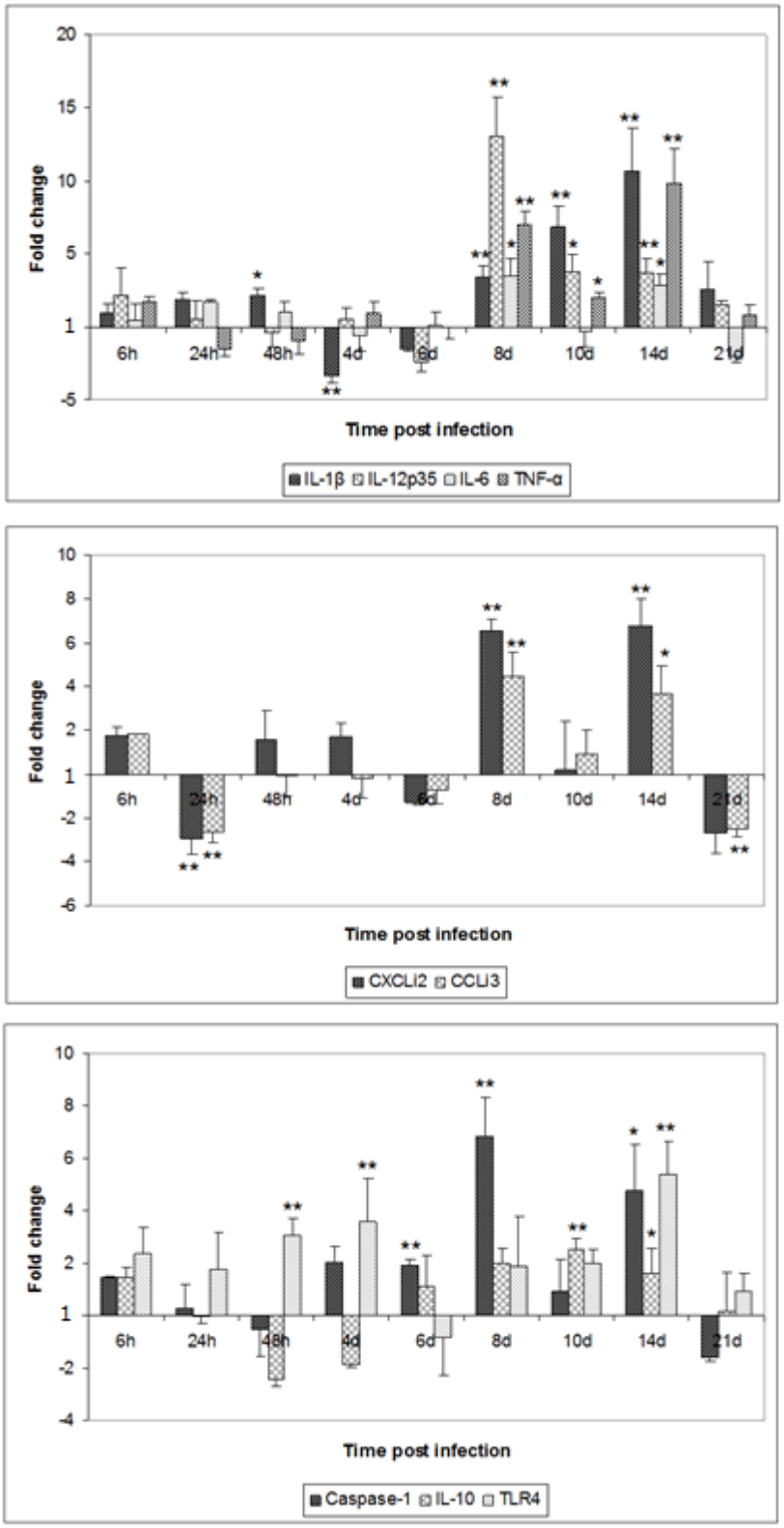


Fig. V-8: Expression of cytokine or chemokine or toll-like receptors in the bursa fabricius of chickens following infection with *C. psittaci*. Results are presented as fold changes in mRNA compared with age-matched mock-infected controls based on samples from four chickens for each time determined by quantitative RT-PCR. Significant differences between *C. psittaci* infected and mock-infected chickens, determined by an unpaired student t test, are indicated by $^{**}P< 0.05$ and $^{*}P<0.1$. The error bars in all figures represent the standard error of the means.

Spleen:

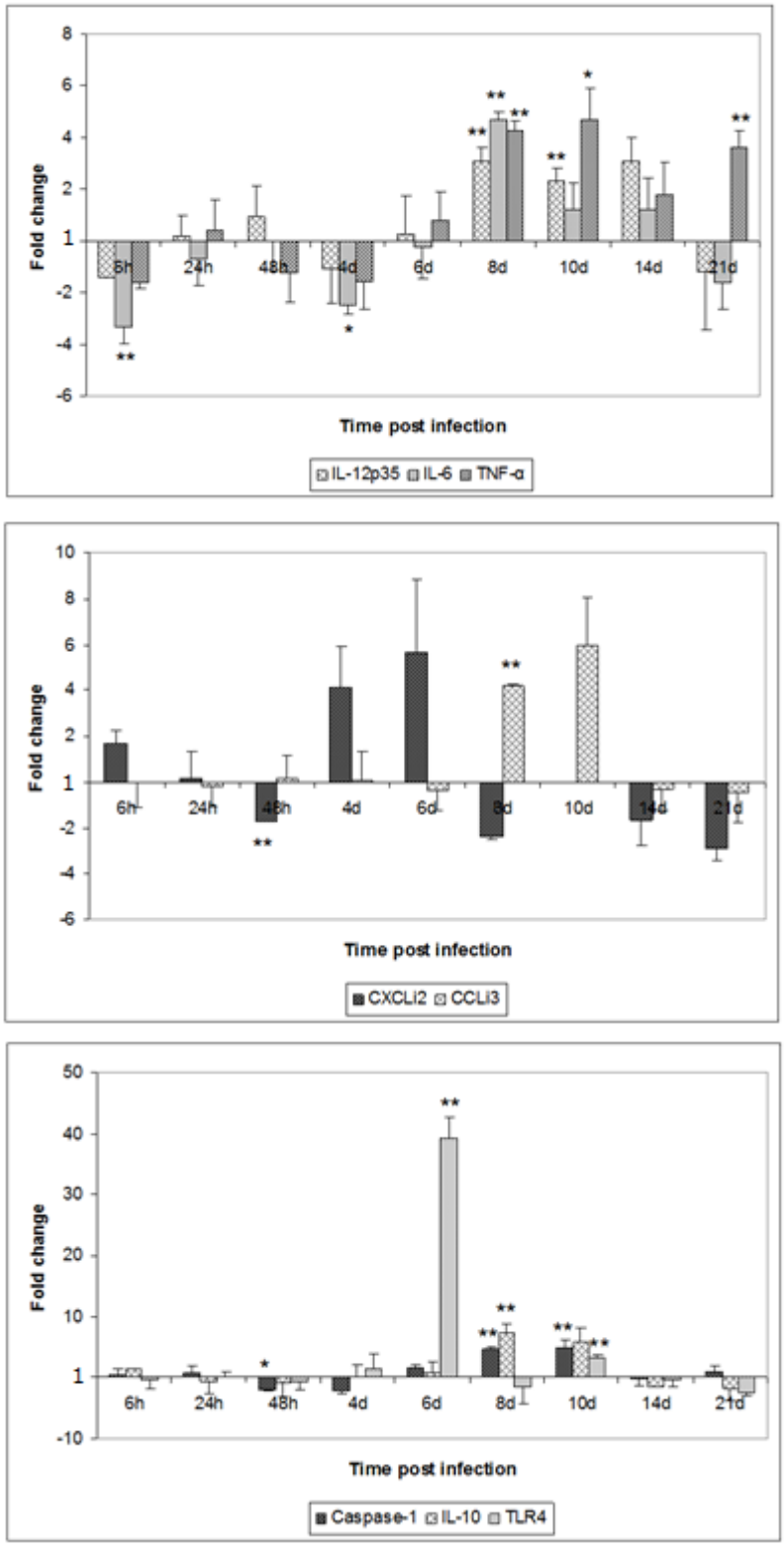


Fig. V-9: Expression of cytokine or chemokine or toll-like receptors in the spleen of chickens following infection with *C. psittaci*. Results are presented as fold changes in mRNA compared with age-matched mock-infected controls based on samples from four chickens for each time determined by quantitative RT-PCR. Significant differences between *C. psittaci* infected and mock-infected chickens, determined by an unpaired student t test, are indicated by $^{**}P< 0.05$ and $^{*}P<0.1$. The error bars in all figures represent the standard error of the means.

4. Discussion

The present study is one of few studies that shed light on the underlying immune response evoked by *C. psittaci* in his natural host, the chicken. Understanding the mechanisms associated with immune responses to *C. psittaci* respiratory infection in early life is important in developing targets for effective vaccination and therapeutic strategies for infection. Identification of the pitoval components of inflammation induced by *C. psittaci* will also facilitate the elucidation of its role in the pathogenesis of acute and persistent infection.

Aerogenous infection with *C. psittaci* is followed by systemic dissemination of the bacteria, probably mediated by monocytes/macrophages, as Vanrompay *et al.* (1995b) showed the presence of *C. psittaci* in blood monocytes recognized by KUL01 in experimentally infected SPF turkeys. *C. pneumoniae* infected monocytic cells are able to activate the integrin adhesion receptor system to invade noninflamed subendothelium and initiate inflammatory processes (May et al., 2003). Interestingly, at 7d p.i. a higher frequency of monocytes/macrophages expresses the MHC class II molecule on their surface in the infected group compared to the mock infected group was observed, indicating that chlamydial antigens entered the monocytes/macrophages through endocytosis. This is in agreement with the results of Ojcius *et al.* (1997), who showed that most *Chlamydia*-containing vacuoles in THP1 cells fuse with lysosomes and compartments expressing MHC class II molecules, suggesting that THP1 cells might present antigens to *Chlamydia*-specific $CD4^+$ T cells. However in this study, no significant difference in the frequency of $CD4^+$ T cells between the infected and the mock-infected group was observed. Remarkably, at 21d p.i. a significant lower frequency of monocytes/macrophages expressing the MHC class II molecule was observed in the infected group compared to the mock infected group. Since only a proportion of monocytes/macrophages and epithelial cells will be infected by *C. psittaci* during an *in vivo* exposure, immune evasion could happen through downregulation of both MHC class I and class II expression evoked by a chlamydia-specific protease called chlamydial protease activity function (CPAF) (Ibana et al., 2011). At every time point investigated, a significant higher frequency of $CD8^+$ T cells in the infected group compared to the mock infected group was observed. As *C. psittaci* is an intracellular pathogen, $CD8^+$ T cells could play a significant role in the protection against this bacterium. However, the role $CD8^+$ cytotoxic T lymphocytes in immunity to *Chlamydiae* have been the subject of less study. Cytotoxic T lymphocytes (CTLs) recognize peptide antigens in the context of MHC class I, expressed on all nucleated cells which direct lyse of the infected cell and production of cytokines such as IFN-γ. The peptides presented to CTLs are derived from the cytoplasmic compartment of the cell (Monaco, 1992). Looking at chlamydial infections, it is unknown if any chlamydial antigens escape from or traffic across the vacuolar membrane. Such antigens could be likely candidates for processing and presentation to CTLs. However, the role of CTLs during *Chlamydiae* infections is in question. During

a *C. trachomatis* infection, both $CD4^+$ and $CD8^+$ T cells are stimulated and secrete IFN-γ (Gondek et al., 2009; Johansson et al., 1997). However, elimination of $CD8^+$ T cells does not compromise protection against a *C. trachomatis* genital infection (Morrison et al., 2000; Frankhauser and Starnbach, 2014). A recent study of Nogeuira (2015) showed that intranasal priming with *C. trachomatis* engages both $CD4^+$ and $CD8^+$ T cells, which are necessary for protection against a genital infection. The protection is largely dependent on IFN-γ secretion by T cells. Another study convincingly showed that *in vivo* depletion of $CD8^+$ T cells in mice abrogates protection upon infection with *C. psittaci*, whereas depletion of $CD4^+$ T cells did not affect protection (Buzoni et al., 1992). The precise role of $CD8^+$ T cells during a *C. psittaci* infection in his natural host must be investigated more in detail. The mucosal surfaces of the respiratory system serve as both entry and transmission route for most pathogens. Therefore, a crucial aspect in designing effective vaccines against *C. psittaci* is the stimulation of immunity at the mucosal sites of the respiratory system. Moreover, T cell migration among the mucosal surfaces is tightly regulated by the interaction of adhesion molecules and chemokine receptors that are differentially expressed on T cells and their target tissues. So, investigating how the immune system respond on infection with *C. psittaci* is of great importance to be able to develop effective therapeutic strategies.

A few recent studies already investigated the immune response elicited by *C. psittaci* in his natural host. A study of Lagae et al. (2015) investigated the chicken macrophage activation and expression of cytokine, chemokine, caspase-1, iNOS and TLR genes during the early phase and mid-cycle period of the developmental cycle of *C. psittaci*. Another study examined the pathology and host immune response following aerogenous infection with *C. psittaci* or *C. abortus* in chickens (Kalmar et al., 2015).

Yin et al. (2013a) investigated the presence of *C. psittaci* in different organs at various time points during a pathogenicity study of low and highly virulent *C. psittaci* isolates in chickens. At a given time point post infection, the presence of this bacterium corresponds well with the observed immune response in this study. In this study, the first host response was examined which is an acute inflammatory or polymorphonuclear leukocyte response, probably initiated by activation of the Toll-like receptor pathway and by other still undefined mechanisms. During the first four days p.i., a significant upregulation of pro-inflammatory cytokines, chemokines and TLR-4 was noticed in the conchae, lungs and airsacs. This aerogenous infection with *C. psittaci* is followed by a systemic dissemination of the infection, as from 6 days p.i. on, a significant immune response is observed in the harderian gland, bursa fabricius and spleen. This systemic infection is mediated by peripheral blood mononuclear cells as alveolar macrophages could migrate through the mucosal barrier and gain access to the systemic circulation. It is possible that *C. psittaci* infection induces rolling and adhesion of macrophages to the non-inflamed vessel wall as previously reported for *C. pneumonia* (May et al, 2003).

Interestingly, the anti-inflammatory cytokine IL-10 was not significant upregulated in the conchae, lungs and airsacs; yet well in the lymphoid organs like the harderian gland, bursa fabricius and spleen. Although, Il-10 suppresses a broad range of inflammatory responses and is an important factor in maintaining homeostasis of overall immune responses (Stober et al, 2005 and Villalta et al, 2011). Unfortunately, little is known about the anti-inflammatory effect of IL-10 during a *C. psittaci* infection. Beeckman et al. (2009) observed exceptionally high levels of IL-10 in *C. psittaci* infected HD11 macrophages at a MOI of 100 after 4h p.i. A study of Yilma et al. (2012) showed that IL-10 inhibits inflammatory mediators in human epithelial cells and mouse macrophages exposed to *C. trachomatis*. Additionally, Gao et al. (2013) reported that IL-10 suppresses expression of inducible co-stimulator-ligand (ICOS-L), an activator of T lymphocytes, on DC in an animal model with C. muridarum lung infection. It is possible that other immunoregulatory molecules like IL-1 receptor antagonist, IL-4, IL-11 and IL-13 act in the conchae, lungs and airsacs to control the pro-inflammatory cytokine response. A recent study has demonstrated that $CD4^+$ Th17 responses are also induced by *Chlamydia* infections (Bai et al., 2009; Scurlock et al., 2011). Th17 cells are differentiated from conventional αβ $CD4^+$ T cells under the influence of different cytokines such as TGF-β, IL-1β, IL-6 and IL-23 (Korn et al., 2009). The pro-inflammatory cytokines secreted by Th17 cells, IL-17A and IL-17F, stimulate the production of cytokines and chemokines involved in neutrophil generation, maturation and mobilization to sites of inflammation. The role of Th17 responses in *Chlamydia* infections is complex; it can promote bacterial clearance but also potentially damage host tissue when improperly controlled (Bai et al., 2009; Zhang et al., 2009; Zhou et al., 2009). Kalmar et al. (2015) also investigated the mRNA expresiion of IL-17 after infection with *C. psittaci* in chickens and noticed a rapid upregulation of this cytokine at 3d p.i. Furthermore, several studies demonstrated an expansion of $Foxp3^+$ T regulatory cells (Tregs) during *Chlamydia* infections (Faal et al., 2006; Gall et al., 2011; Marks et al., 2007). Intriguingly, infection with *C. muridarum* subverted the suppressive role of Tregs on the immune systems into the promotion of Th17 differentiation from conventional $CD4^+$ T cells but also converted themselves into pro-inflammatory Th17 cells. Further elucidating of the immune mechanisms that effectively regulate Th17 response may help to understand the pathogenesis of *Chlamydia*.

Remarkably, during the *C. psittaci* infection several cytokines and chemokines are significant downregulated in various organs. It has been previously shown that several effector molecules, translocated by the type III secretion system (T3SS) of many Gram-negative bacterial pathogens inhibit or subvert PAMP-directed innate immune reponses to promote infection. For example, four effector molecules of *Yersinia*, YopH, YopE, YopT and YpkA antagonize phagocytosis. YopJ inhibits nuclear factor kappa B (NF-κB) and mitogen-activated protein (MAP) kinase signaling pathways and can induce a pro-inflammatory mode of cell-death associated with caspase-1 activation. (Viboud and Bliska, 2005; Philip

and Brodsky, 2012). The effector molecule IpaH4.5 of *Shigella flexneri* is an E3 ubiquitin ligase capable of directly regulating the host inflammatory response by inhibiting the NF-κB signaling pathway (Wang et al. 2013). How the effector molecules of *C. psittaci* regulate the host immune response during infection has not yet been uncovered. A study of Prantner and Nagarajan (2009) showed that inhibition of the T3SS of *C. muridarium* led to decreased IL-6, IL-1β and CXCL10 levels. In comparison, inhibition of the T3SS of *C. psittaci* evoked a significant lower mRNA expression of IL-1β, IL-6, LITAF, IL-12p35, caspase-1, macrophage inhibitory factor (MIF), IL-10, CXCLi1, CXCLi2, CCLi3, IL-16, TLR-2, TLR-3, TLR-4, TLR-5, TLR-7 and TLR-21 in chicken macrophages during the mid-phase of the developmental cycle. Intriguingly, during the early-phase a significant upregulation was noticed for CXCLi1, IL-16 and TLR-4 genes (unpublished results). The T3SS is also required to initiate inflammation via activation of caspase-1 or NF-κB-dependent genes. For example, the *Pseudomonas aeruginosa* T3S effector ExoS activates both TLR2 and CD14/TLR4 signaling (Epelman et al. 2004). *Shigella* IpaB and *Salmonella* SipB (homologous to the chlamydial CopB) colocalize with caspase-1 and are necessary for its activation (Prantner and Nagarajan, 2009).

Only one pattern recognition receptor (PRR), TLR4, was investigated in this study with a significant upregulation in the conchae, lungs, harderian gland, bursa fabricius and spleen during infection. However in the airsacs, TLR4 was significant downregulated at 8d p.i. Contrary, Kalmar et al. (2015) reported a significant upregulation of TLR4 at 7d and 14d p.i. in the thoracic airsacs. Chlamydial LPS and chlamydial heat-shock proteins are ligands for the TLR4 receptor. Recent studies describe that TLR2 plays a significant role as a pattern recognition receptor for and is involved in early secretion of pro-inflammatory mediators, while TLR4 has a protective role (Agrawal et al., 2011; Massari et al., 2013; Prebeck et al., 2001; Beckett et al. 2012). In this study, TLR4 is significant upregulated in every organs examined except the airsacs, but it is possible that TLR4 is not responsible for the observed production of pro-inflammatory mediators. To our knowledge, only two studies has yet investigated the role of the pattern recognition receptors during a *C. psittaci* infection (Lagae et al., 2015; Kalmar et al., 2015). Different studies revealed not only a role for TLRs but also for NOD-like receptors (NLRs) and inflammasomes during a chlamydial infection (Shimada et al., 2012).

In conclusion, to our knowledge, this is one of the few studies wherein *in vivo* immune response during a *C. psittaci* infection was studied in his natural host, the chicken. However, a comprehensive understanding about the vital mechanisms controlling *C. psittaci*-caused pathological sequelae remains lacking. It will be an important future goal to identify the mediators, the PRRS and the cells involved in the host immune response during a *C. psittaci* infection and better define how these factors interact with each other; findings that would have important implications to C. *psittaci* vaccine design and development.

Chapter VI

General discussion and perspectives

General discussion and perspectives

Chlamydia psittaci is an obligate intracellular bacterium causing respiratory disease and mortality in birds. The disease is endemic in many species of wild birds, but also in poultry like chickens, turkeys, ducks and geese (Newman et al., 1992; Vanrompay et al., 1997; Verminnen et al., 2008). Recently, *C. psittaci* has been found more often in chickens (Yin et al., 2013a; 2013b). In order to prevent mortality, reduced feed intake and carcass condemnation at slaughter, antibiotic treatments are still being used in farms to control these infections.

C. psittaci is also a zoonotic pathogen. It causes parrot fever or psittacosis in humans after aerogenic transmission of the bacteria from diseased birds (Andersen and Vanrompay, 2000). Several reports described the transmission of *C. psittaci* from birds to humans, particularly in high-risk individuals such as veterinarians, poultry workers, ped bird owners, bird breeders, taxidermists or traders (Hinton et al., 1993; Vanrompay et al., 1995a; Heddema et al., 2006; Harkinezhad et al., 2007; Verminnen et al., 2008; Beeckman and Vanrompay, 2009; Larouceau et al., 2009). However, reports on *C. psittaci* zoonotic transmission from chicken farms are rare (Gaede et al., 2008; Dickx and Vanrompay, 2011). Recently, we examined the prevalence of *C. psittaci*, atypical *Chlamydiaceae* and their possible zoonotic transmission on 19 Belgian chicken farms. ACC were not detected in the chickens or in the human samples. However, 18 out of 19 farms were positive for *C. psittaci* by both culture and PCR. *C. psittaci ompA* genotypes A and D were discovered among the chickens. Looking at the humans, 29 out of 31 (93.5%) were positive for *C. psittaci* by culture and PCR. Genotypes A, D and a mixed infection with genotypes C and D were found. This study shows that *C. psittaci* infections in chickens are, at least in Belgium, more prevalent than originally thought but it also emphasizes the importance of *C. psittaci* as a zoonotic pathogen. About 86.2% of the positive farmers reported yearly medical complaints potentially related to psittacosis and four of them currently experienced respiratory disease. About 12.5% of the farmers mentioned that they had pneumonia after starting to keep chickens. It has been described that established workers gained a certain level of protection against clinical disease. New employees more often become ill after contact with *C. psittaci* (Newman et al., 1992; Hinton et al., 1993). However, specific measures for controlling *C. pittaci* infections are recommended for all persons at risk. People should be adequately informed about the risk and on protective procedures. They should be trained how to use protective equipment which include suitable clothing, gloves, disinfecting soap and a full-face mask or at least mask that covers mouth and nose as a full face mask is not always easy to wore during work. Collective protective measures such as ventilation and frequent cleaning to prevent aerosol accumulation and cross-contamination between the different barns or different rooms in a slaughterhouse are also needed (Deschuyffeleer *et al.,* 2012). Accurate diagnostic monitoring in poultry and reporting of infections by physicans is required. In this respect, an increased

awareness of occupational physicians is also crucial. Despite the European Union's obligation to assess any biohazard in the workplace, knowledge on *C. psittaci* is still relatively undeveloped and a specific risk assessment has not been composed yet. So, there is still room for improvement.

C. psittaci is endemic in the poultry industry causing economic damage, although specific calculations of economic losses are unavailable. Large epidemiological studies have been performed but they can never prove the economic impact of *C. psittaci* in poultry. Intervention studies are needed for this purpose. During such an intervention study, one is able to calculate the economic benefit obtained by using a specific preventive strategy. However, we have no *C. psittaci* preventive strategies, as there is no vaccine and the currently used antibiotics are also effective against other respiratory pathogens. As this bacterium has an important zoonotic potential, a way to control or even eradicate this bacterium must be found. Currently, antibiotics are the only weapon against chlamydiosis. Tetracyclines are the drugs of choice for avian chlamydiosis. Enrofloxacin might also be used but it is less effective and more expensive. Moreover, it belongs to the fluoroquinolones and some countries (like for instance The Netherlands and Belgium) forbid the use of these antibiotics in farm animals as they are frequently used in human medicine. Prophylactic use of antibiotics is out of the question because the risk of creating tetracycline resistant *C. psittaci* strains and the risk of antibiotic residues in poultry meat. Antibiotics have also become environmental contaminants, which might have a negative impact on our ecosystem (Pouliquen et al., 2008). So far, tetracycline resistant *C. psittaci* strains have not been described in the literature. However, tetracycline resistant *Chlamydia suis* strains have been found in pigs, probably as an undesirable consequence of the long term use of antibiotics as growth promoters (in feed) in these animals. The latter has been forbidden since 1997 (Lenart et al., 2001; Dugan et al., 2004).

Vaccination would be a good strategy to prevent *C. psittaci* infections as it could lead to a better control of respiratory disease and to reduction of related financial losses (cost of antibiotics, reduced economic performance). Moreover, it could reduce of the use of antibiotics (less antibiotics residues in environment and meat). At present, a vaccine against *C. psittaci* is not yet commercially available, although there are already several commercial vaccines against *C. abortus* in ruminants and *C. felis* in cats. These vaccines are based on the use of killed or live attenuated bacteria. Especially, the live vaccines offer good protection and significantly reduce the shedding of infective organisms. However, concerns remain over the safety of using live-attenuated vaccines, particularly for zoonotic pathogens. Moreover, immunopathology can occur when using whole organism based chlamydial vaccines (Mabey et al., 2014).

Vaccination studies on the development of a protective DNA vaccine, based on the major outer membrane protein (MOMP) of *C. psittaci* have been performed in turkeys. These studies revealed good results, as the vaccine was able to significantly prevent severe clinical signs and lesions and significantly reduced bacterial replication and excretion. Unfortunately, full protection could not be achieved, if albeit possible (Verminnen et al., 2005; Loots et al., 2006). The major outer membrane protein (MOMP) is an immunodominant protein which represents the majority of the surface exposed proteins and contains serovar-specific neutralizing epitopes. It has been shown that this protein is the most protective antigen (Caldwell et al., 1981; Hatch et al., 1981), so vaccine studies focused on MOMP. However, major efforts have been made to develop an effective vaccine with MOMP, although no vaccine is yet commercially available.

During this thesis, a recombinant *C. psittaci* vaccine was developed and evaluated. Results will be presented later on, as the patent filing is not yet completed.

Another approach to combat *C. psittaci* is ovotransferrin which is anti-microbial protein capable of binding iron needed by bacteria to survive in the host (Valenti et al., 1982; Valenti et al., 1983). A study of Beeckman et al. (2007) demonstrated that ovotransferrin was able to inhibit actin polymerization and internalization of *C. psittaci* into chicken macrophages. To further explore the possible role of ovotransferrin as a prophylactic use, Van Droogenbroeck et al. (2008, 2011) set up two different *in vivo* experiments in turkeys. These experiments show that two administration rounds of ovotransferrin by aerosol based on the *C. psittaci* infection waves protect turkeys against *C. psittaci* infection and other respiratory infection outbreaks. However, the use of ovotransferrin cannot avoid the occurrence of these infections but clinical signs were not observed and antibiotics weren't administered during the experiment. Moreover, such mild infections could stimulate the immune system in such a way that clinical signs are not present. It is worthwhile to invest in both approaches (vaccine and ovotransferrin) to reduce the use of antibiotics in the poultry industry.

Developing knowledge on the protective immune mechanisms to *C. psittaci* infections is crucial for vaccine development.

Therefore, in this thesis, the *in vitro* and the *in vivo* immune responses elicited by *C. psittaci* in the host were examined as well as the role of the T3SS in this complex mechanism. Upon entry in the host, *C. psittaci* infects and colonizes the epithelial cells and the macrophages of the respiratory tract. *C. psittaci* is able to invade and even replicate within macrophages, which are part of the innate immune system.

Former studies mainly focused on the adaptive immune response against *C. trachomatis*, *C. muridarum*, *C. pneumoniae*, and *C. caviae*, whereas it is the early innate immune system that provides

the first stage of defence against those bacteria upon entering the mucosal lining and establishing a productive infection. Very few studies have investigated the innate immune system of the avian respiratory tract (Ariaans et al., 2008; Sarmento et al., 2008; Wang et al., 2011) and only one study has examined innate immunity to *C. psittaci* in its natural host cell, the respiratory epithelial cell or avian macrophage (Beeckman et al., 2010). However, the use of natural host cells in *in vitro* experiments is important, as earlier demonstrated by Roshick et al. (2006). They evaluated the growth of *C. trachomatis* and *C. muridarum* in various human and mouse cell lines and concluded that the role of different antichlamydial effector mechanisms are best performed in matched host/chlamydial sets (Roshick et al., 2006). In this thesis, we therefore focused on the innate immune response elicited by *C. psittaci in vitro* in chicken and human macrophages, but also *in vivo*.

For the *in vitro* studies, matched host cell lines were used, the avian monocyte/macrophage HD11 cell line and the human monocyte/macrophage THP1 cell line. *C. psittaci* actively infect macrophages by inducing actin recruitment at the site of invasion by using the sophisticated T3SS. *C. psittaci* can also enter the macrophage through phagocytosis. Phagocytosis of bacteria is a normal function of macrophages. They patrol the tissues of the body and ingest and destroy unwanted pathogens. Some pathogens such as *Chlamydiae*, have acquired the ability to survive and replicate within macrophages after they have been phagocytosed. According with other infection studies using monocytes/macrophages, a limited replication or a barely survival of *C. psittaci* was observed (Beeckman et al., 2010). Our observation suggest that *C. psittaci* might use the macrophage as a vehicle to transmigrate through the mucosal barrier present in the lungs and subsequently have access to the lymphatic system and the systemic circulation to spread to the other tissues present throughout the body and establish a systemic infection (Moazed et al., 1998; Gieffers et al., 2004).

The macrophage senses the presence of the bacteria through his pattern recognition receptors located extracellular and intracellular of the cell. In chapter III, it was for the first time observed that the avian intracellular receptor TLR21 was significant upregulated after infection with *C. psittaci* in avian macrophages. Moreover, a significant upregulation of TLR3, TLR4, TLR5 and TLR7 was also observed. This activation of several PRRs leads to the activation of signaling cascades, resulting in the production of several chemokines, pro- and anti-inflammatory cytokines. However, the precise immune mechanisms involved in pathogenesis to *C. psittaci* infection, especially in the receptor signaling and downstream activation and suppression during the innate phase of infection initiating the adaptive immune responses remains largely unknown. In this thesis, it is clear that the pro-inflammatory cytokines IL-1β and IL-6 are strong upregulated after infection. This two cytokines and are part of the acute phase response which maintain the inflammation localized to the site of infection, clear cellular debris and has a role in the chemotaxis (Schneider et al., 2001; Kaiser et al., 2004). Nevertheless,

different chemokines such as CXCLi1, CXCLi2, CCLi3 and IL-16 were investigated. CXCLi1 and CXCLi2 binds with the cognate receptor CXCR1 but have different roles. CXCLi1 and CXCLi2 induce the migration of heterophils and monocytes, respectively (Poh et al., 2008). According to the study of Beeckman et al. (2010) the CXCLi1 mRNA expression was upregulated to a higher level than CXCLi2 following infection with *C. psittaci*. Furthermore, CCLi3 was also high upregulated which also attracts heterophils to the place of infection. Elevation of these chemokines support the presence of *C. psittaci* in heterophils which could be the result of active phagocytosis by heterophils once they arrive, or because *C. psittaci* actively invades those cells (Johns et al., 2009) (Fig. VI-1).

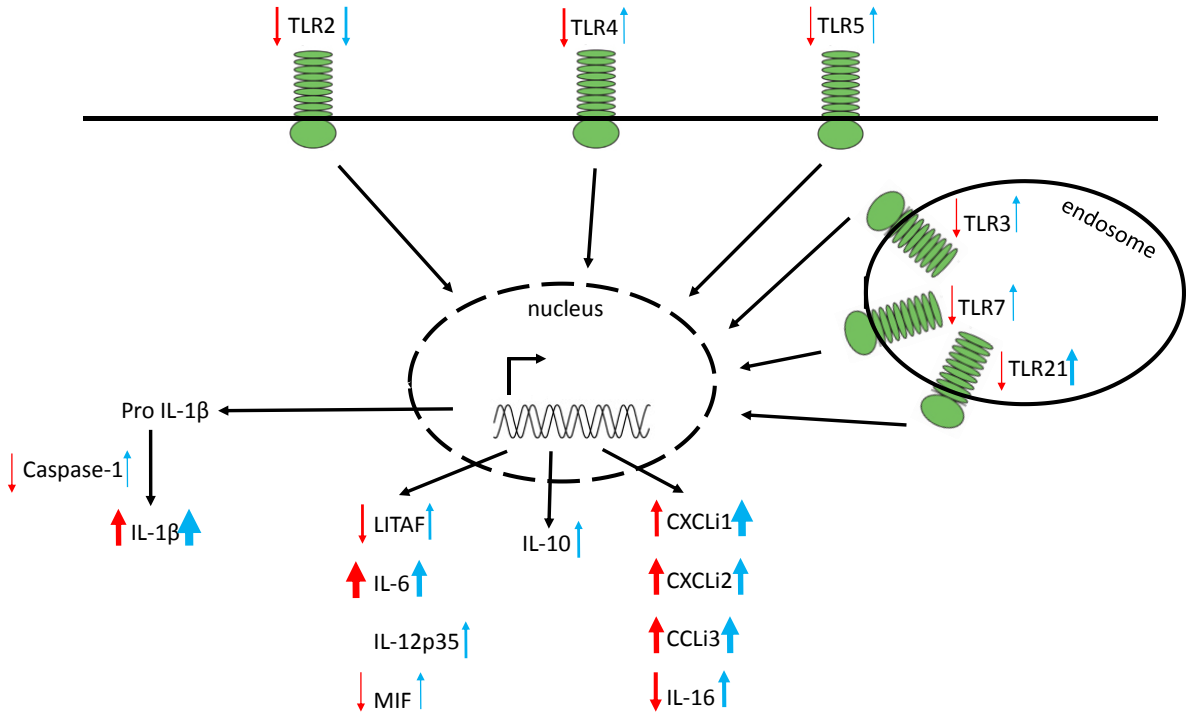


Fig. VI-1: Schematic overview of the investigated innate immune response in *C. psittaci* infected HD11 cells. Red and blue arrows indicate the up- or downregulation of several genes investigated during the early- and mid developmental cycle of *C. psittaci*, respectively. The thickness of the arrows shows the relative expression of each gene investgated.

However, only chemo attractants such as CXCLi1, CXCLi2, CCLi3 and IL-16 were investigated after *C. psittaci* infection of macrophages. It would be interesting to examine the leukocyte extravastion or diapedesis *in vivo* during infection with *C. psittaci*. This process involves several steps like chemoattraction, rolling adhesion, tight adhesion and transmigration. Several studies indicate that *C. pneumoniae* is recognized as a causative agent of respiratory tract infections and implicated as a potential risk factor or a cause of several extrapulmonary diseases such as multiple sclerosis, atherosclerosis and Alzheimer disease (Grimaldi et al., 2003; Janczak and 2015; Shima et al., 2010). *C. pneumonia* infected monocytic cells showed enhanced transmigration and attach to the endothelium

via VLA-4, LFA-1 and MAC-1 to invade noninflamed subendothelium and initiate inflammatory processes. Thus, *C. pneumoniae* has the potential to activate the integrin adhesion receptor system in monocytic cells and can actively contribute to the monocyte recruitment to sites of atherosclerotic lesions (May et al., 2003). Moreover, the disease atherosclerosis is known as the most common pathological change observed in blood vessels of captive psittacine birds (Pees et al., 2006). A recent study of Pilny et al. (2012) indicated that infection of *C. psittaci* and high plasma cholesterol concentration may be risk factors for developing atherosclerosis in pet psittacine birds. However, how *C. psittaci* contribute to the disease atherosclerosis must be elucidated. This outcome will also be very relevant for the public health as *C. psittaci* is a zoonotic agent.

Chlamydiales have developed different mechanisms on the molecular and cellular level to circumvent recognition and activation of the innate immunity. Because of their need to dedifferentiate into RBs before replication, these bacteria need to control the immune system to have enough time to complete their biphasic lifecycle. The T3SS is a highly conserved multiprotein syringelike structure that translocate different bacterial effector proteins in the cytoplasm of the host cell. Many reports addressed the importance of the role of the T3SS in inflammation of different pathogens such as *Salmonella* spp. (Miao et al., 2006) *Yersinia* spp. (Shin and Cornelis, 2007), *Shigella* spp. (Suzuki et al., 2007), *Pseudomonas* spp. (Franchi et al., 2007; Miao et al., 2008; Sutterwala et al., 2007), and *Burkholderia* spp. (Sun et al., 2005) through the activation of caspase-1 and NF-κB downstream genes such as IL-6, Mip-2 and monocyte chemoattractant protein 1. As the T3SS constitute an important virulence-associated mechanisms which is commonly spread among gram-negative bacteria, those small organic molecules such as INP0007 blocking the secretion of TTS system substrates have been proposed as a new class of antimicrobial agents called virulence blockers (Keyser et al., 2008). However, many of those virulence blockers tend to be toxic for the host cells. A study of Ur-Rehman et al. (2012) has screened a library of 58 salicylidene acylhydrazides to identify *Chlamydia* growth. Only twelve compounds were not toxic to the cell. At the end, two compounds were selected as promising treatment of Chlamydial infections. In the future, the specific role of the different effector proteins translocated by the T3SS on the innate immune response will be further elucidated. Therefore, *C. psittaci* mutants lacking an effector protein could be used (Wang et al., 2013).

In chapter V, the in vivo immune response elicited by *C. psittaci* in chickens was investigated. During the first 48 hours, a significant pro-inflammatory response (IL-1β, IL-6, TNF-α, IL-12, CXCLi2, CCLi3, caspase-1, TLR-4) was observed in the conchae. A shift of the same pro-inflammatory response was observed to the lungs at 4 dpi. As the airsacs are anatomically closely related to the lungs, an immune response was also observed at 4 dpi. with the exception of IL-12, the chemokines and TLR4 which were not significantly upregulated. Later on in the infection (8 dpi, 10dpi, 14dpi and 21dpi), an initiating of

the immune response was seen in the harderian gland, bursa fabricius and spleen, indicating the presence of a systemic infection from 8 dpi. A significant upregulation of TLR4 was noticed in the spleen and the harderian gland. Again, no significant upregulation of the chemokines investigated were observed in the harderian gland. These results resembles well with the observations seen in HD11 cells. But it must be noticed that in this study the innate immune response of not only macrophages but all cell types present in the respective organs was investigated.

In chapter IV, an elongated shape of some macrophages were observed after infection with *C. psittaci*. A study of McWhorter et al. (2013) showed that elongation of human macrophages induce polarization toward a M2 phenotype. Macrophages can polarize toward a spectrum of phenotypes which include the classical pro-inflammatory M1 and the alternative anti-inflammatory pro-healing M2 activation state (Mosser and Edwards, 2008). Those macrophages remain relatively plastic which mean that they are able to taken in multiple phenotypes depending on the environment. M1 macrophages possess bactericidal properties, especially against intracellular pathogens by releasing several pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, chemokines and ROS, whereas M2 macrophages support the growth of those pathogens (Kahnert et al., 2006). *Francisella* induce a M2 polarization in macrophages by inducing the production of IL-4 and IL-13, which support their replication and survival (Shirey et al., 2008). However, a study of Gracey et al. (2013) showed that *C. muridarum* does not induce murine macrophage polarization during intracellular infection. Furthermore, the polarized macrophages have a different capacity to control this bacterium, with M2 macrophages being permissive to *Chlamydia* growth and M1 macrophages being able to control *C. muridarum* through the induction of persistence. Intriguingly, M2 macrophages are more susceptible to *Chlamydia* infection than non-polarized macrophages, probably due to the increased expression of the mannose receptor on those macrophages facilitating the uptake of *Chlamydia* (Murray and Wynn, 2011). In this way, it may take longer for M2 polarized macrophages to activate antimicrobial mechanisms than their unstimulated macrophages. Interestingly, a study of Jupelli et al. (2013) have demonstrated a shift in the numbers of M1 macrophages to M2 macrophages during *C. pneumoniae* lung infection. As we observed several elongated macrophages during intracellular infection with *C. psittaci*, it would be worthwhile to characterize macrophage – *C. psittaci* interactions in the context of macrophage polarization over a long period *in vivo*. However, we can already speculate about this polarization during *C. psittaci* infection as we have investigated several cytokines and chemokines during infection produced by macrophages. We observed a high pro-inflammatory cytokine production which is associated with M1 macrophages, which initiates adaptive immune T cell responses and tissue inflammation (Qin et al., 2012). However, looking at the *in vivo* study, a significant upregulation of the

anti-inflammatory cytokine IL-10 is observed from 8 days post infection, suggesting a shift from some M1 macrophages to M2 macrophages.

Remarkably, it was observed that the lowest MOI yields a significant larger inclusion when compared with other MOIs in THP1 macrophages. This indicates that *C. psittaci* is able to sense the neighborhood and the amount of EBs. This inter-bacterial communication is called quorum sensing which is a widespread phenomenon in the microbial world that has important implications in the coordination of population-wide responses in several bacterial pathogens (Bassler, 2002). This system allow bacteria to recognize the density of the bacterial population by sensing and measuring the accumulation of specific small signal molecules that bacteria of the community secrete. When the amount of the accumulated signals in the environment is sufficient, signaling pathways are activated that alter bacterial gene expression. Quorum sensing makes bacteria able to behave as a community and to perform tasks which would be impossible for individuals bacteria such as overcoming defense and immune system and establishing infections (Rutherford and Bassler, 2012; Fazli et al., 2014). Unfortunately, quorum sensing has not yet been investigated in Chlamydial species.

In general, pro-inflammatory cytokines such as IL1-β, IL-6, TNF-α and IL-8 were detected in macrophages after a *C. psittaci* infection (Fig VI-2). Remarkably, the protein IL-12 was not found in the supernatant of activated THP1 macrophages. However, IL-12 bridges together with IL-18 the infection with the IFN-γ production in the innate immune response. Recognition of pathogens by macrophages normally induces secretion of chemokines and IL-12 and which attract NK cells to the site of inflammation and promotes IFN-γ synthesis in these cells (Otani et al., 1999). Many reports indicate that dendritic cells and macrophages can produce IFN-γ (Frucht et al., 2001). However, no IFN-γ production was detected in the supernatant during a *C. psittaci* infection in human macrophages. Although, IFN-γ seems to be important during chlamydial infection as demonstrated by the enhanced bacterial levels IFN-$\gamma^{-/-}$ or IFN-γ receptor$^{-/-}$ mice, or mice treated with anti-IFN-γ antibodies, compared with controls (Cotter et al., 1997; Rottenberg et al., 1999; Johansson et al., 1997; Ito and Lyons, 1999). However, the mRNA products of IL-12 were detected in activated chicken macrophages and in the conchae, lungs, airsacs, bursa and spleen of infected chickens. It could be that this cytokine together with IL-18 induce the production of IFN-γ in T cells and NK cells *in vivo* during a *C. psittaci* infection in chickens.

The human homologue TLR9 was also significant upregulated in the early- and mid-phase of the infection cycle of *C. psittaci* in THP1 macrophages (Fig. VI-2). TLR9 recognizes bacterial DNA containing unmethylated CpG motifs and an upregulation of TLR9 mRNA was also observed during an intranasal *C. pneumonia* infection (Han et al., 2004). However, a study of Rothfuchs et al. (2004) describes that

TLR4 deficient bone marrow derived macrophages (BMDM) showed increased Chlamydial progeny concomitant with IFN-α expression, but that was not the case for TLR2$^{-/-}$, TLR6$^{-/-}$and TLR9$^{-/-}$ BMDM. Further investigation of the recognition by and the initiation of the signal cascade of TLR9 and its avian homologue TLR21 during *C. psittaci* infection would be interesting.

In our study, TLR2 was not or significantly downregulated during infection, suggesting that TLR2 does not play a role during *C. psittaci* infection. However, *C. pneumoniae* activates NF-κB in a TLR2 dependent manner in 293HEK cells (Prebeck et al., 2001). Indeed, TLR2 seems to be important for bacterial clearance and survival during a *C. pneumonia* infection *in vivo* and *in vitro* (Rodriguez et al., 2006; Beckett et al., 2012). In contrast to the study of Beckett et al. (2012) and Netea et al. (2002), TLR4 seems to be important in the battle against *C. psittaci* as it is significant upregulated in the human and avian macrophages as well as in the spleen, the bursa fabricius, the harderian gland, the lungs and the conchae during a *C. psittaci* infection in chickens.

As TLR4 and MyD88 doubly deficient macrophages could still produce IFN-α/γ in response to *C. pneumoniae* infection, this implicates the existence of a TLR independent pathway in *C. pneumoniae* recognition (Rothfuchs et al., 2004). Therefore, several other PRRs such as NLRP3, ASC, RIG-I and NOD-2 were investigated during *C. psittaci* infection in human macrophages. Remarkably, those intracellular receptors were significant upregulated during the early phase of the infection and not during the mid-cycle (Fig. VI-2). The mechanisms behind this exceptionally observation must still be investigated.

In our study NOD-2 was only significantly upregulated early in the infection cycle. Several studies report that both NOD-1 and NOD-2 play important roles during a *C. pneumoniae* infection (Opitz et al., 2005; Shimada et al., 2009). Coordinated and sequential activation of TLRs and NODs signaling pathways will be necessary for an efficient immune response against chlamydial infections. It is likely that TLRs might be important for initial activation and NOD proteins for a sequential, prolonged activation of the target cells by intracellular *Chlamydiae*.

Another important family investigated is the NLR family which are involved in the recognition of DAMP leading to the assembly of inflammasomes, which in turn leads to the generation of active caspase-1, a requirement for the production of mature IL-1β and IL-18. The mRNA expression of the inflammasome NLRP3/ASC was significantly upregulated during infection with C. *psittaci* in human macrophages (Fig. VI-2). Furthermore, the protein IL-1β was detected in the supernatant of the activated macrophages. *In vivo*, the mRNA expression of IL-1β and caspase-1 was significantly upregulated in different organs such as the conchae, lungs, airsacs, harderian gland and bursa fabricius, indicating an important role for the NLR family during *Chlamydial* infection.

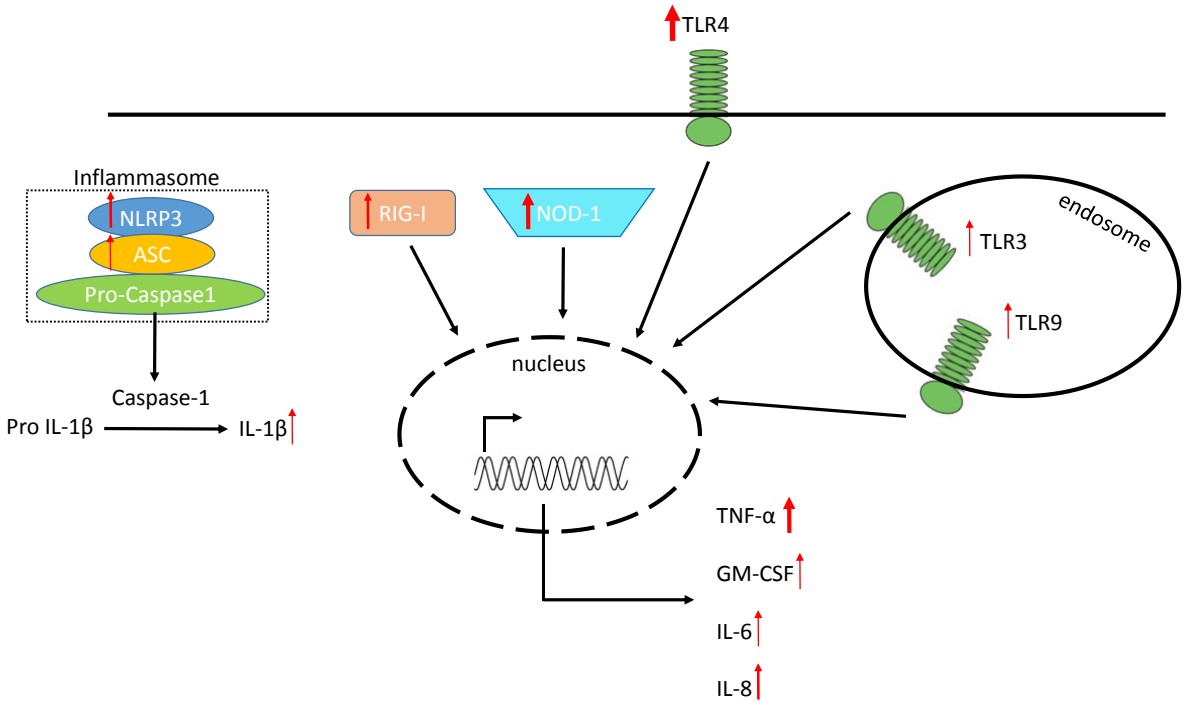


Fig. VI-2: Schematic overview of the investigated innate immune response in *C. psittaci* infected THP1 cells. Red arrows indicate the upregulation of several genes and proteins investigated during the early-, mid- and late developmental cycle of *C. psittaci*. The thickness of the arrows shows the relative expression of each gene/protein investgated.

In conclusion, we emphasized the endemic nature of *C. psittaci* in chickens and its zoonotic potential by describing the transmission of this bacteria from infected chickens to the farmers. Furthermore, the results presented in this thesis has led to a better understanding of the immunological mechanism mediated by *C. psittaci* in his natural host, indicating that *C. psittaci* initiates a pro-inflammatory response and is able to establish a systemic infection.

Summary

The focus of this thesis was on the avian Gram-negative obligate intracellular bacterium, *Chlamydia psittaci*. *C. psittaci* causes avian chlamydiosis and is mainly spread by inhalation of aerosols of nasal secretion or fecal material containing the infectious particle. It is known that *C. psittaci* can infect 400 species, covering 30 different bird orders (Kaleta and Taday, 2003). In the poultry industry, *C. psittaci* causes a lowered egg production, higher mortality and carcass condemnation (Grimes and Wyrick, 1991). The bacteria can be transmitted to humans resulting in psittacosis in which the symptoms can vary from inapparent to flu-like symptoms and even pneumonia (Harkinezhad et al., 2007; Harkinezhad et al., 2009; Van Droogenbroeck et al., 2009; Verminnen et al., 2006).

After inhalation, primary replication of *C. psittaci* takes place in the epithelial cells and macrophages of the respiratory tract. Later on, the bacteria can be found in epithelial cells and macrophages of the lower respiratory tract. And in severe cases, the infection can become systemic whereby *C. psittaci* is present in blood macrophages spreading to various tissues throughout the body. Macrophages are an important part of the innate immune system and capable of engulfing and killing pathogens. But probably their most important function is to recruit other immune cells to the place of infection by the elaboration of chemokines. Macrophages can also activate the adaptive immune response by presenting the antigen. As macrophages play such an important role in clearing pathogens, it is rather unique that *C. psittaci* as well as other *Chlamydiaceae* are able to survive and even replicate within those cells.

Besides illustrating that *C. psittaci* is endemic in the Belgian chicken industry and his zoonotic nature, this thesis focuses in the investigation of the innate immune response *in vitro* elicited by *C. psittaci* in chicken and human macrophages but also *in vivo* in his natural host, the chicken. This obtained knowledge was used to design and evaluate a recombinant *C. psittaci* vaccine.

Chapter I of this thesis gives an overview of the history, classification and biology of *C. psittaci* in animals and humans. Furthermore, this chapter also focuses on immunology and chlamydial vaccine development.

In **Chapter II**, the prevalence of *C. psittaci* and atypical *Chlamydiaceae* and their zoonotic transmission was investigated on 19 Belgian chicken farms. Pharyngeal swabs from chickens and farmers were investigated for the presence of *C. psittaci* using a combination of nested PCR, outer membrane protein A (*omp*A) genotype-specific quantitative real-time PCR and cell culture. The chickens of 18 out of 19 farms were positive for *C. psittaci* by culture and PCR and genotype A and D were detected. Of the investigated farmers were 93.5% positive for *C. psittaci* by culture and PCR and genotypes A, D and a mixed infection with genotypes C and D were discovered. No atypical *Chlamydiaceae* were detected.

This study shows that *C. psittaci* infections are endemic in the Belgian chicken industry and possess a huge zoonotic risk to all people in close contact.

Chapter III was dedicated to studying the innate immunity initiated by *C. psittaci* in chicken macrophages (HD11 cells). *C. psittaci* engages an initimate relation with avian respiratory epithelia cells and macrophages. The mRNA expression of different cytokines (IL-1β, IL-6, MIF, TNF-α, IL-12p35, IL-10, GM-CSF), chemokines (CXCLi1, CXCLi2, CCLi3, IL-16), caspase-1, iNOS and toll-like receptors (TLRs) (TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-21) were investigated during the early (2h-8h p.i.) and mid-cycle (12h-18h p.i.) phase of the developmental cycle of the highly virulent *C. psittaci* strain 92/1293. In general, an augmentation of transcript levels for all genes investigated was detected, especially during mid-cycle.

In **chapter IV**, we examined the relationship between *C. psittaci* and the human macrophage (THP-1 cells), with the emphasis on internalization, survival, replication, T3SS and the innate immune system. The development of *C. psittaci* in human macrophages is very similar to the infection cycle in epithelial cells. Bacterial replication was quantified through titration of the cell culture supernatant and immunofluorescence assays were set up to examine the inclusion size and the inclusion forming units per ml. Rapid early entry was observed followed by an increase in the average area per inclusion between 24h and 48h p.i. which is likely to be the exponential growth of *C. psittaci*. At 48h p.i., lysis of the host cell was observed with the release of newly formed elementary bodies (EBs). At 15 min p.i., actin recruitment was observed and the continuous expression of the effector protein IncA was detected, supporting the assumption of a preloaded T3SS. During infection, the other effector proteins investigated, SctC and SctW were also observed. In addition, the gene expression level of a panel of pattern recognition receptors (TLR-1, TLR-2, TLR-3, TLR-4, TLR-6, TLR-9, NLRP3, ASC, RIG-I, NOD-2) were investigated during the early- (2h, 4h, 8h), mid- (12h, 18h, 24h) and late- (36h, 48h, 72h) phase of the developmental cycle of *C. psittaci* to evaluate how this bacterium is recognized by his host cell. Upon this recognition, a signal cascade is induced resulting in the production of several cytokines and chemokines. Different cytokines and chemokines such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IFN-γ, TNF-α and GM-CSF were investigated in the supernatant of *C. psittaci* infected THP1 cells during the mid- and late-phase of the developmental cycle using a Multi-Analyte ELISArray Kit. The cytokines IL-1α, IL-2, IL-4, IL-10, IL-12, IL-17α and IFNγ were not detected, indicating that those cytokines were not expressed during infection with *C. psittaci* in human macrophages. In addition, high concentrations of the cytokines IL-1β and TNF-α and the chemokine IL-8 was noticed throughout all time points measured, whereas the cytokine IL-6 and GM-CSF was rather late in the infection observed from 48h p.i. and 36h p.i. on, respectively.

The aim of **chapter V** was to elucidate the *in vivo* immune response evoked by *C. psittaci* in his natural host, the chicken. Excretion of *C. psittaci*, chlamydial antibody detection in sera, blood immune cells and the mRNA expression levels of different cytokines (IL-1β, IL-6, IL-10, IL-12, TNF-α, caspase-1), chemokines (CXCLi2 and CCLi3) and one Toll-like receptor (TLR-4) were investigated in different organs (conchae, lungs, airsacs, harderian gland, bursa fabricius and spleen) at different time points p.i. (6h, 24h, 48h, 4d, 6d, 8d, 10d, 14d and 21d). A higher frequency of monocytes/macrophages expressing the MHC II molecule and cytotoxic $CD8^+$ T cells were observed in chickens infected with C. psittaci. Several cytokines, chemokines and TLR-4 were also significantly upregulated during infection in several organs. Remarkably several cytokines and chemokines were also significantly downregulated, especially at late time points. This study can contribute on the elucidation on how *C. psittaci* interact with his host, leading to the developing of targets for effective vaccination and therapeutic strategies for infection.

Finally, **Chapter VI** describes the conclusions of this thesis and future perspective worthwhile to investigate.

Samenvatting

Deze doctoraatsthesis spitst zich toe op *Chlamydia psittaci*, een gram-negatieve obligaat intracellulaire bacterie voor vogels. *C. psittaci* veroorzaakt chlamydiosis bij vogels. Het wordt voornamelijk verspreid door inhalatie van besmette aerosols afkomstig van nasale secreties of fecaal materiaal. *C. psittaci* kan 400 vogelspecies, behorend tot 30 verschillende ordes infecteren (Kaleta and Taday, 2003). Vooral in de pluimvee-industrie leiden *C. psittaci* infecties tot aanzienlijke verliezen ten gevolge van een verlaagde ei productie, verhoogd sterftecijfer en karkassen die niet geschikt zijn voor consumptie (Grimes and Wyrick, 1991). De bacterie kan ook mensen besmetten. Humane psittacosis heeft een heel divers klinisch beeld variërend van onbeduidend tot grieperige symptomen of longontsteking (Harkinezhad et al., 2007; Harkinezhad et al., 2009a; Van Droogenbroeck et al., 2009a; Verminnen et al., 2006).

Na inhalatie van de bacterie treedt primaire replicatie op in de epitheliale cellen en macrofagen van de bovenste luchtwegen. Daarna kan de bacterie gedetecteerd worden in de epitheliale cellen en macrofagen van de onderste luchtwegen. In ernstige gevallen kan de infectie systemisch worden. Hierbij is *C. psittaci* aanwezig in bloed macrofagen die de bacterie verspreiden naar verschillende weefsels in het lichaam. Macrofagen zijn cellen die een belangrijk deel vormen van het aangeboren immuunsysteem. Deze cellen kunnen pathogenen fagocyteren. Maar hun belangrijkste taak is het aantrekken van andere immuuncellen naar de plaats van infectie door middel van chemokines. Daarnaast kunnen macrofagen het adaptief immuunsysteem activeren door antigenpresentatie. Doordat macrofagen een heel belangrijke rol spelen in het opruimen van pathogenen, is het uniek dat *C. psittaci* en andere leden van de *Chlamydiacea* familie in staat zijn om te overleven en zelfs te repliceren in deze cellen.

Enerzijds toont deze doctoraatsthesis aan dat *C. psittaci* een zoönotisch karakter heeft en endemisch is in de Belgische kippenindustrie. Anderzijds ligt de focus ook op het verwerven van kennis omtrent de aangeboren immuunrespons tijdens een *C. psittaci* infectie. Dit werd zowel *in vitro* onderzocht in kippen- en humane macrofagen als *in vivo* in zijn natuurlijke gastheer, de kip. De verworven kennis werd gebruikt om een recombinant vaccin tegen *C. psittaci* te contstrueren en te avlueren.

Hoofdstuk I van deze doctoraatsthesis geeft een overzicht weer van de geschiedenis, classificatie en biologie van *C. psittaci* in dier en mens. Daarnaast ligt de nadruk ook op immunologie en de ontwikkeling van een vaccine tegen *Chlamydiae*.

In **hoofdstuk II** werd de prevalentie van *C. psittaci* en atypische *Chlamydiaceae* en hun zoönotische transmissie onderzocht op 19 Belgische kippenbedrijven. Faryngeale swabs van kippen en de pluimveehouders werden onderzocht op de aanwezigheid van *C. psittaci* door gebruik te maken van

een combinatie van nested PCR, outer membrane protein A (*ompA*) genotype-specifieke kwantitatieve real-time PCR en celcultuur. De kippen van 18 bedrijven waren positief voor *C. psittaci* in celcultuur en nested PCR. In deze bedrijven werd genotype A en D gedecteerd. 93,5% van de pluimveehouders waren positief voor *C. psittaci* in celcultuur en nested PCR. Hierbij werd genotype A, D en een gemengde infectie met genotype C en D ontdekt. Atypische *Chlamydiaceae* werd niet gedecteerd op de kippenbedrijven. Deze studie toont aan dat *C. psittaci* endemisch aanwezig is in de Belgische kippenindustrie. Daarnaast bezit deze bacterie een groot zoönotisch karakter voor alle mensen die regelmatig dicht in contact komen met deze kippen.

In **hoofdstuk III** werd de aangeboren immuunsysteem onderzocht gedurende een *C. psittaci* infectie in kippenmacrofagen (HD11 cellen). *C. psittaci* gaat een hechte verbintenis aan met hun eukaryote gastheercel, namelijk epitheelcellen en macrofagen. Verder gebruikt *C. psittaci* een T3SS om effector proteïnen te transloceren in het cytoplasma van zijn gastheercel. Hierdoor wordt de pathogenese van *C. psittaci* bevorderd. De mRNA expressie van verschillende cytokines (IL-1β, IL-6, MIF, TNF-α, IL-12p35, IL-10, GM-CSF), chemokines (CXCLi1, CXCLi2, CCLi3, IL-16), caspase-1, iNOS and toll-like receptors (TLRs) (TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-21) werden onderzocht gedurende de vroege- (2h-8h p.i.) en de mid-fase (12h-18h p.i.) van de ontwikkelingscyclus van de hoge virulente *C. psittaci* stam 92/1293. Algemeen werd een stijging van de transcriptieniveaus van alle genen waargenomen, voornamelijk tijdens de mid-fase van de ontwikkelingscyclus.

In **hoofdstuk IV** werd de relatie tussen *C. psittaci* en de humane macrofaag (THP-1 cel) onderzocht met de nadruk op de internalisatie, overleving, replicatie, T3SS en het immuun systeem. De ontwikkeling van *C. psittaci* in humane macrofagen is sterk vergelijkbaar met de ontwikkelingscyclus waargenomen in epitheliale cellen. Bacteriële replicatie werd gekwantificeerd door middel van titratie van het supernatans van geïnfecteerd cellen. Daarnaast werden immunofluorescence assays gebruikt om de inclusie grootte en de inclusie vormende units per ml te onderzoeken. Een snelle intrede werd gevolgd door een stijging in de grootte van de inclusie tussen 24h en 48h p.i. Deze observatie is hoogstwaarschijnlijk te wijten aan de exponentiële groei van *C. psittaci*. Lyse van de gastheercel werd geobserveerd 48h na de infectie. Hierdoor komen nieuw gevormde elementaire lichaampjes vrij (EBs). Actinpolymerisastie na 15 min na infectie en de continue expressie van de effector proteïne IncA werden gedecteerd. Deze observaties ondersteunen het vermoeden dat het T3SS reeds geladen is met een aantal effector proteïnen in het begin van de infectie. Gedurende de infectie werden nog andere effector proteïnen geobserverd zoals SctC en SctW. Daarnaast werd de genexpressie van een aantal pattern recognition receptors (TLR-1, TLR-2, TLR-3, TLR-4, TLR-6, TLR-9, NLRP3, ASC, RIG-I, NOD-2) onderzocht gedurende de vroege- (2h, 4h, 8h), mid- (12h, 18h, 24h) en late- (36h, 48h, 72h) fase van de ontwikkelingscyclus van *C. psittaci* om na te gaan hoe deze bacterie wordt herkend door zijn

gastheercel. Na deze herkenning word een signal cascade geïnitieerd wat resulteert in de productie van verschillende cytokines en chemokines. Verschillende cytokines en chemokines zoals IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17α, IFN-γ, TNF-α en GM-CSF werden onderzocht in het supernatans van geïnfecteerde THP1 cellen gedurende de mid- en late-fase van de ontwikkelingscyclus van *C. psittaci* door gebruik te maken van een Multi-Analyte ELISArray Kit. De cytokines IL-1α, IL-2, IL-4, IL-10, IL-12, IL-17α and IFNγ werden niet gedecteerd. Deze resultaten suggereren dat deze cytokines niet tot expressie komen tijdens een infectie met *C. psittaci* in humane macrofagen. Daarnaast werden wel hoge concentraties van de cytokines IL-1β en TNF-α en the chemokine IL-8 gedecteerd gedurende alle tijdspunten. Verder werden de cytokine IL-6 en GM-CSF ook gedecteerd laat in de infectie, namelijk vanaf 48h and 36h respectievelijk.

Het doel van **hoofdstuk V** was om de *in vivo* immuunrespons tijdens een *C. psittaci* infectie in zijn natuurlijke gastheer, de kip te onderzoeken. Excretie van *C. psittaci*, detectie van antilichamen tegen *Chlamydiae* in het serum, bloed immuuncellen en de mRNA expressie van verschillende cytokines (IL-1β, IL-6, IL-10, IL-12, TNF-α, caspase-1), chemokines (CXCLi2 and CCLi3) en één toll-like receptor (TLR-4) werd onderzocht in verschillende organen (conchae, longen, luchtzakken, harderse klier, bursa fabricius en milt) op verschillende tijdspunten postinfectie (6h, 24h, 48h, 4d, 6d, 8d, 10d, 14d and 21d). Een hogere frequentie van monocyten/macrofagen die de MHC klasse II molecule tot expressie brengen en $CD8^+$ T cellen werden geobserveerd in kippen geïnfecteerd met *C. psittaci*. Verschillende cytokines, chemokines and TLR-4 werden significant opgereguleerd gedurende de infectie in verscheidene organen. Daarnaast was het opmerkelijk dat verschillende cytokines en chemokines ook significant waren neergereguleerd, voornamelijk tijdens de late tijdspunten. Deze studie draagt bij tot het ontrafelen hoe *C. psittaci* interageert met zijn gast. Deze kennis kan leiden tot de ontwikkeling van een effectief vaccine en therapieën tegen een *C. psittaci* infectie.

Hoofdstuk VI beschrijft een aantal besluiten van deze doctoraatsthesis alsook mogelijkheden voor verder onderzoek.

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Curriculum Vitae

PERSOONLIJKE GEGEVENS

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Belgische nationaliteit
Rijbewijs klasse B

OPLEIDINGSGEGEVENS

2015-heden	Leerkracht wetenschappen Sint-Barbaracollege Gent
2014-2015	GON leerkracht op Sint-Lodewijk Kwatrecht (BuSo, opleidingsvorm 4)
2009-2014	Assistente van de vakgroep Moleculaire Biotechnologie (Immunologie en Biotechnologie van de Dierlijke Cel) aan de Faculteit Bio-ingenieurswetenschappen van de Universiteit Gent
2012-2013	Attest Specifieke Lerarenopleiding
2007-2009	Master Biomedische Wetenschappen optie Immunologie en Infectie aan de Faculteit Geneeskunde en Gezondheidswetenschappen van de Universiteit Gent
2004-2007	Academische Bachelor Biomedische Wetenschappen aan de Faculteit Geneeskunde en Gezondheidswetenschappen van de Universiteit Gent
2005-2007	Moderne Talen -Wetenschappen aan het Sint-Bernarduscollege te Oudenaarde
2003-2005	Moderne Talen - Wiskunde aan het Sint-Bernarduscollege te Oudenaarde

TALENKENNIS

Nederlands	Moedertaal
Engels	Zeer Goed
Frans	Basis
Duits	Basis

COMPUTERSKILLS

Goede kennis van Microsoft Word, Excel, Powerpoint.
Basis kennis van SPSS (statistical software)

ONDERWIJSERVARING

Organiseren en begeleiden van practica (Ugent):

- Immunologie: 1ste master Bio-ingenieurswetenschappen: optie Cel-en gentechnologie
- Biotechnologie van de Dierlijke Cel: 1ste master Bio-ingenieurswetenschappen: optie Cel-en gentechnologie
- Biochemische analysetechnieken: 1ste master Bio-ingenieurswetenschappen: optie Chemie en Bioprocestechnologie, optie levensmiddelenwetenschappen en voeding
- Biochemie en Moleculaire Biologie: 2de bachelor Bio-ingenieurswetenschappen

Theoretische lessen geven (Ugent):

- Immunologie: 1ste master Bio-ingenieurswetenschappen: optie Cel-en gentechnologie
- Biotechnologie van de Dierlijke Cel: 1ste master Bio-ingenieurswetenschappen: optie Cel-en gentechnologie
- Biochemische analysetechnieken: 1ste master Bio-ingenieurswetenschappen: optie Chemie en Bioprocestechnologie, optie levensmiddelenwetenschappen en voeding
- Chemie (2de graad) / Fysica (2-3de graad) / Biologie (3de graad) / Wetenschappelijk Werk (2-3de graad)

Secundair onderwijs:

- GON leerkracht
- → Sint-Lodewijkschool, Kwatrechtsesteenweg 168 te Wetteren.
  BuSO – type 4 – OV4
- Leerkracht chemie, biologie, fysica, aardrijkskunde
  ➔ Sint-Barbaracollege Gent; 2de graad

ERVARINGEN – ONDERZOEKSSKILLS

Vakgroep Moleculaire Biotechnologie (Faculteit Bio-Ingenieurswetenschappen, Universiteit Gent)

- Schrijven van wetenschappelijke rapporten en artikels
- Rapporteren en presenteren van behaalde resultaten
- Laboratoriumervaring: cel- en weefselcultuur, bacteriecultuur, DNA en RNA extractie, PCR en (q)RT-PCR, micro-arrays, kloneren, plasmide opzuivering, recombinante eiwit expressie (transfectie van eukaryote cellen), SDS-PAGE en immunobloting, ELISA, immunofluorescentie technieken, Flow Cytometrie, proefdieren experimenten (set-up, staalname), werkzaam in een bioveiligheid klasse 3 laboratorium, gebruik van klasse 2 en klasse 3 organismen (Chlamydiaceae)

Thesis studenten:

2009-2010: **Bert Persan.** Epidemiologisch onderzoek naar *Chlamydophila psittaci* en invloed van het type III secretiesysteem op de immuunrespons.
2010-2011: **Neil Saad.** The role of the type III secretion system of *Chlamydia psittaci* in human macrophages. Masterproef voorgedragen tot het behalen van de graad van Master in de Bio-ingenieurswetenschappen: Cel-en Gentechnologie
2010-2011: **Delphine De Sutter.** *Chlamydia psittaci* vaccinatie bij kippen. Masterproef voorgedragen tot het behalen van de graad van Bachelor in de Chemie, afstudeerrichting Biochemie
2010-2011: **Anoesjka Steenbeke.** *Chlamydia psittaci* vaccinatie bij kippen.
2011-2012: **Bieke Soen.** Onderzoek naar de aangeboren immuunresposn van *Chlamydia psittaci* in humane macrofagen. Masterproef voorgedragen tot het behalen van de graad van Master in de Bio-ingenieurswetenschappen: Cel-en Gentechnologie
2011-2012: **Ellen Audenaert.** Detectie en epidemiologie van *Chlamydia psittaci* op Belgische kippenbedrijven. Masterproef voorgedragen tot het behalen van de graad van Master in de Bio-ingenieurswetenschappen: Cel-en Gentechnologie
2011-2012: **Eveline Jonckheere.** Analyse van de immuunrespons bij SPF kippen na infectie met *Chlamydia psittaci*. Masterproef voorgedragen tot het behalen van de graad van Master in de Bio-ingenieurswetenschappen: Cel-en Gentechnologie

PUBLICATIES

Schautteet K, De Clercq E, Jönsson Y, Lagae S, Chiers K, Cox E, Vanrompay D, 2012. Protection of pigs against genital *Chlamydia trachomatis* challenge by parenteral or mucosal DNA immunization. Vaccine. 30 (18) 2869-81.

Yin L, Kalmar ID, Lagae S, Vandendriessche S, Vanderhaegen W, Butaye P, Cox E, Vanrompay D, 2013. emerging *Chlamydia psittaci* infections in the chicken industry and pathology of *Chlamydia psittaci* genotype B and D strains in specific pathogen free chickens. Vet Microbiol. 162 (2-4) 740-49.

Yin L, Lagae S, Kalmar I, Borel N, Pospischil A, Vanrompay D, 2013. Pathogenicity of low and highly virulent *Chlamydia psittaci* isolates for specific pathogen free chickens. Avian Diseases. 57 242-247.

Lagae S, Kalmar I, Laroucau K, Vorimore F, Vanrompay D, 2014. Emerging *Chlamydia psittaci* infections in chickens and examination of transmission to humans. J Med Microbiol. 63 399-407.

Lagae S, Vanrompay D, 2015. Innate immune response in avian macrophages eeicited by *Chlamydia psittaci*. Vlaams Dierengeneeskundig tijdschrift. 84 133-141.

Lagae S, Dumont A, Vanrompay D, 2015. Examination of the *in vivo* immune response elicited by *Chlamydia psittaci* in chickens. Vet Immunol Immunopathol. In press.

MEETINGS

Met orale presentatie

9th German Chlamydia Workshop. Ascona, Switzerland. 22-25 februari 2011. *"Influence of the Chlamydia psittaci type III secretion system on the innate immune response of chicken macrophages."*
8th Annual Amsterdam Chlamydia meeting (AACM). Hotel Mercure Amsterdam aan de Amstel, Amsterdam, Nederland. 9 december 2011. *"Influence of the Chlamydia psittaci type III secretion system on the innate immune response of chicken macrophages."*

Met poster presentatie

25th Annual Meeting of the European Macrophage en Dendritic Cell Society (EMDS). Brussels, Belgium, 22-24 september 2011. *"Influence of the Chlamydia psittaci type III secretion on the innate immune response of chicken macrophages."*

Zonder presentatie

Summer Course on Infection and Immunity. Faculty Veterinary Medicine, Ghent, Belgium. 29 en 30 september 2009
6th Annual Amsterdam Chlamydia meeting (AACM). Hotel Mercure Amsterdam aan de Amstel, Amsterdam, Nederland. 17 november 2009

OPLEIDINGEN

Seminarie Welzijn en milieu, Gent, Belgium. 17 september 2009
FACSAria III Operator Training, BD Erembodegem, Belgium. 31mei-4 juni 2010.
Cursus Real-time PCR: Biogazelle leuven, Belgium. oktober1-2, 2012

HOBBY'S

Verenigingsleven: Speelpleinwerking (Kruishoutem); 3 jaar leiding
KSJ – VKSJ –KSA Kruishoutem; 5 jaar leiding

Andere: lezen, mountainbiken, naaien en reizen